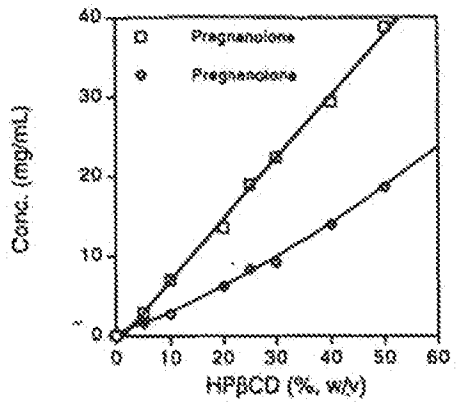

Figure 1—Phase–Solubility profile for pregnanolone (A$_L$-type) and pregnenolone (A$_P$-type) as a function of 2-hydroxypropyl-β-cyclodextrin (HPβCD) concentration.

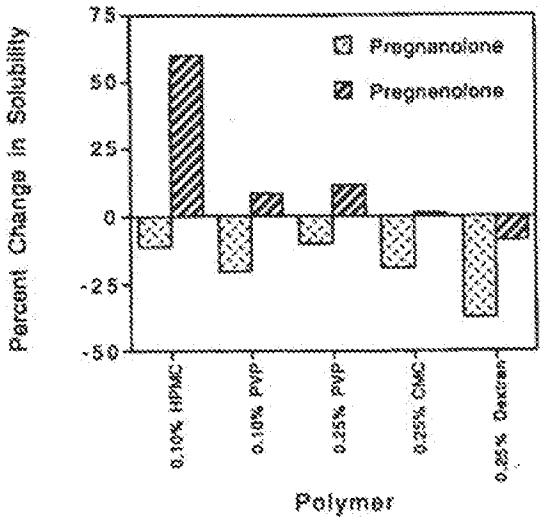

Figure 2—Effect of various water-soluble polymers on the solubilization of pregnanolone and pregnenolone by a 10% w/v solution of HPβCD.

approximately 4760 M^{-1} in the case of pregnenolone and 9000 M^{-1} in the case of pregnanolone. These values are in good agreement with other structurally related steroids.[38] Solid complexes of pregnanolone or pregnenolone with HPβCD were prepared and contained 101.3 and 56 mg/g, respectively.

One approach that has been suggested to enhance drug solubilization in cyclodextrin is the use of small quantities of pharmaceutical polymers such as HPMC, PVP, CMC, and dextrans. The method of preparation of these cyclodextrin systems include heating the drug, polymer, and cyclodextrin under autoclave conditions. As illustrated in Tables 2 and 3 and Figure 2, the hydrophilic polymers did not provide improved solubilization in the case of pregnanolone. On the other hand, HPMC and to a lesser extent PVP improved the solubility of pregnenolone in HPβCD with best results with 0.10% HPMC which generated an increase in solubility of 60%. These compounds are thought to interact with cyclodextrins and their guests to form thermodynamically stable ternary complexes.[47] Data supportive of this model include published information showing an interaction between cyclodextrins and various cellulose derivatives,[48] NMR studies demonstrating direct intermolecular interactions, and diffusion studies. In the latter, the ternary complex (which required heating at autoclave conditions to form) for various system was found to diffuse more slowly through semipermeable cellulose membranes than did unconditioned (solutions which were not heated) systems containing the three components (T. Loftsson, unpublished results).

Table 4—Effect of Intravenous Pregnanolone and Pregnenolone on Sedation (Percent Sedated) and Sleeping Time in Male Mice

Dose (μmol/kg)	Pregnanolone % Sedated	Pregnanolone Sleeping Time (min)	Pregnenolone % Sedated	Pregnenolone Sleeping Time (min)
0 (Vehicle)	0	0	0	0
12.5	17	0.3 ± 0.3	0	0
25	100	8.2 ± 1.7	0	0
75	100	>15	0	0
100	100	>15	0	0

The initial pharmacological assessment of the two complexes used a mouse model to quantitate the nature, onset, and duration of the anesthetic reaction. As illustrated in Table 4, pregnenolone was inactive as either an induction or maintenance agent at doses as high as 100 μmol/kg (32 mg/kg). Pregnanolone, conversely, exerted significant activity with iv administration sedating 100% of animals at 25 μmol/kg (8 mg/kg). These results are consistent with previously published data for pregnanolone administered in an organic cosolvent. Gyermek et al. found that the minimum anesthetic dose for pregnanolone was 2.5 mg/kg when the drug was administered iv in dimethyl sulfoxide.[27] Comparisons of the current results for pregnenolone with historical assessments are more problematic. Gyermek et al. reported that pregnenolone is inactive when administered ip using a paradigm in which the drug was given as a suspension in CMC.[27] Under these conditions, it is possible that the poor solubility of pregnenolone may have masked potential activity. The ability to solubilize pregnenolone in aqueous HPβCD has allowed for a direct measure of the activity of the drug through an iv administration route (the first such determination for this steroid). Results from the current evaluations strongly point to a lack of intrinsic pharmacological activity rather than a limitation due to pharmaceutical parameters (poor aqueous solubility or poor complex dissociation) in defining the spectrum of anesthetic activity. This suggestion is bolstered by the magnitude of the equilibrium constant for pregnenolone, which would indicate a dynamic complex half-life of ≪1 ms, meaning that complexation should not significantly reduce the bioavailability of the steroid.[49] Pregnanolone, which is associated with a complexation constant of almost twice that of pregnenolone, demonstrates potent and rapid action with induction times on the order of a few seconds after iv administration.

Importantly, these data strengthen the argument that the anesthesia provoked by pregnanolone is the result of a specific receptor interaction. Traditionally, anesthetic action has been related to physicochemical properties of the agent examined including both lipid solubility (Meyer–Overton hypothesis) and molecular volume.[50] Such considerations do not seem to be involved with the actions of the steroids currently under investigation. Small structural modifications such as the introduction of a double bond result in only limited changes in log P and other properties (Table 1) but major alterations in pharmacological potency, i.e., pregnanolone is a highly potent anesthetic agent while pregnenolone is devoid of pharmacological activity. These data would suggest that a high degree of structural specificity is required for action, pointing to a receptor-mediated event.[51,52] Similar conclusions were reached with alfaxalone and Δ^{16}-alfaxalone. As in the present circumstance, alfaxalone is a highly potent anesthetic steroid while the Δ^{16}-alfaxalone derivative lacks any significant activity. While some evidence suggests that the reason for this difference is related to membrane bilayer effects, the

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Table 5—Effect of Intravenous or Intraperitoneal Pregnanolone Dose on Induction Times in Male and Female Rats

Male		Female	
Dose (mg/kg)	Induction Time (s)	Dose (mg/kg)	Induction Time (s)
	Intravenous		
1.4	no induction	11	79.67 ± 4.8*
2.2	4.3 ± 0.4	46	60.27 ± 2.5
2.9	3.7 ± 0.2	69–207	<3.0
5.7	4.6 ± 0.7		
11.0	5.6 ± 0.6		
46–155	<3.0		
	Intraperitoneal		
46	500 ± 31	107	898 ± 42*
76	558 ± 48	138	634 ± 49*
107	465 ± 42		
138	295 ± 19		
276	495 ± 37		

* For equivalent doses, asterisks denote significant ($p < 0.05$) differences between males and females.

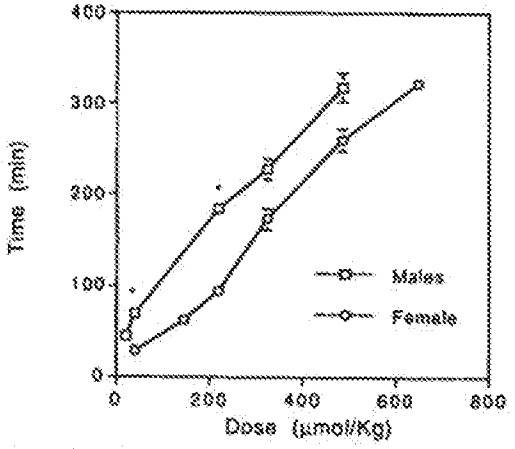

Figure 3—Effect of various intravenous doses of pregnanolone on sleeping time in male and female rats. Asterisks denote a significant ($p < 0.05$) difference between males and females.

strongest evidence suggests interaction with a specific (GABA) receptor.[50,38,54]

Several rat studies were completed to establish and characterize the pharmacological potency of the pregnanolone•HPβCD complex including iv, ip, and po dosing to male and female animals. The induction of anesthesia is reported in Table 5. As indicated, induction times subsequent to iv drug administration were generally quite rapid in both males and females and were generally not dose-dependent. A statistically slower induction in females was observed at doses lower than 69 mg/kg (i.e., at a dose of 11 mg/kg, it required nearly 80 s for induction in female rats while similarly treated male rats were sedated within 6 s of drug administration). Sleeping time was highly correlated with dose of drugs, as illustrated in Figure 3. The response curves for males and females were parallel and there was a tendency for males to be more sensitive to the effects of the steroid than females. Significant differences in sleeping time were observed in the lower portion of the dose range but disappeared at higher doses. Data from this study also suggested that pregnanolone is a more potent anesthetic agent than alfaxalone but that this increased potency was sex-related. On a molar basis, pregnanolone produces a 4.5-fold longer sleeping time than did alfaxalone over a range of doses in male animals while it was almost 50% less potent in females.[41]

Intraperitoneal administration generated induction times which were significantly longer after those associated with

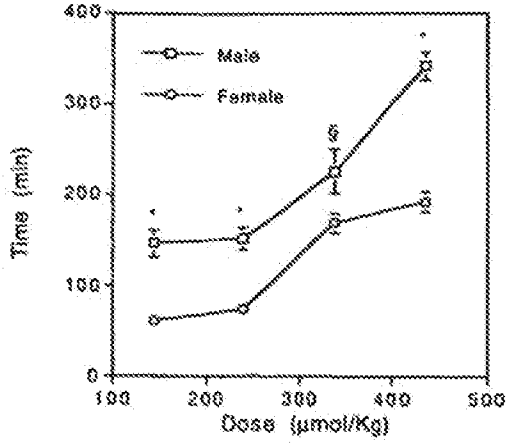

Figure 4—Effect of various intraperitoneal doses of pregnanolone on sleeping time in male and female rats. Asterisks denote a significant ($p < 0.05$) difference between males and females. At the 337 μmol/kg dose (§), $p = 0.052$.

iv administration. As in the case of iv dosing, significant sex differences associated with ip induction were apparent, with males exhibiting significantly shorter times to sedation than females (Table 5). At a dose of 107 mg/kg, for example, nearly 15 min was required for induction in female rats while similarly treated male rats were sedated within 8 min of drug administration. Similarly, sleeping times were dose-dependent for both males and females, with male animals being significantly more sensitive to the effects of the steroid than female rats (Figure 4). These results are interesting, especially when related to the action of alfaxalone. Use of alfaxalone iv over a dose range of 1.5 to 48 mg/kg in the rat was not associated with a significant sex difference.[9] On the other hand, ip data suggested a profound effect. Specifically, Fink et al. found that alfaxalone was more potent in females than in males and that this difference was age-related and could be abolished by administration of estrogens to the male rats.[55] The current data revealed that pregnanolone also exerts a sex difference which tends to be more apparent after ip than iv dosing but one in which males were more sensitive than females. The ip data indicated that, on average, males slept almost twice as long as females for a given dose. When the data is compared to that of alfaxalone, ip dosing in males gave significantly longer sleeping times for pregnanolone than for alfaxalone (by approximately 3-fold) while females were more sensitive to alfaxalone than pregnanolone.[41] The reason for the more potent action of pregnanolone in males as compared to females is not known but this steroid is known to express wide species and sex variations.[29]

Oral dosing of the pregnanolone complex to male rats did not result in induction or loss of righting at the highest nonlethal doses administered (704 μmol/kg, 221 mg/kg). In females, doses of 110.5 and 221 mg/kg resulted in induction times of 23.6 and 26 min, respectively, and sleeping times of 135.3 and 212.6 min, respectively. As in the males, the next higher does (1438 μmol/kg) proved to be lethal. Thus, in the case of po administration, females were more sensitive to males, in contrast to parenteral dosing.

Toxicology assessments were made using an acute lethality paradigm. As illustrated in Figure 5, pregnanolone was significantly more toxic to males than females, with LD_{50} values of 355 and 548 μmol/kg, respectively. The higher toxicity is consistent with the higher pharmacological activity. The LD_{50} of pregnanolone when administered ip to males was determined to be 465 μmol/kg. The value for females could not be determined since the highest dose examined (433 μmol/kg or 136 mg/kg) caused no deaths in the treatment group. Oral doses of the pregnanolone complex of 1438 μmol/kg in

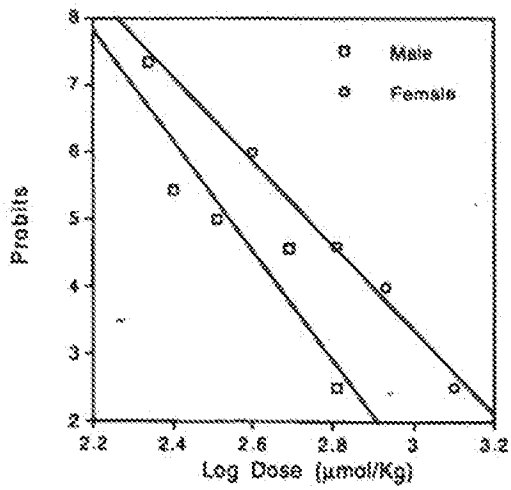

Figure 5—Acute lethality of intravenously administered pregnanolone in male and female rats.

females or 1028 μmol/kg in males resulted in 100% mortality of the treated animals.

In conclusion, water-soluble complexes of pregnanolone and pregnenolone were prepared and tested in animal models of anesthesia. While complexes of pregnenolone were inactive as iv anesthetics (confirming historical ip data), pregnanolone exerted highly potent action. Interestingly, while alfaxalone exerts more efficacious action in females as opposed to males, pregnanolone appears to be more potent in males after parenteral administration. Conversely, oral administration of pregnanolone suggested greater potency in females as opposed to males. The pharmacological action of pregnanolone was exerted rapidly after iv administration, indicating that the cyclodextrin complexation in no way mitigated the bioavailability of the drug. These data suggest that HPβCD may be a useful vehicle for steroid anesthetics in both experimental procedures[5,7,8] as well as in potential clinical development. Support for this statement comes from the solubilizing power of the excipient and its safety in various species including humans. Emulsion-based formulations of pregnanolone used in clinical trials were configured to contain 4 mg/mL. Using the cyclodextrin-based technology described herein, a similar system could be configured by using HPβCD concentrations between 5 and 10% w/v. At human anesthetic doses of pregnanolone (0.5 to 1.0 mg/kg),[39] a 4 mg/mL/10% w/v HPβCD formulation would generate a total HPβCD dose of 12.5–25 mg/kg, which is in the range of that already using in advanced clinical trials.[39]

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CLINICAL REPORT

Pyodermatitis–Pyostomatitis Vegetans: A Clinical Course of Two Decades with Response to Cyclosporine and Low-dose Prednisolone

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Pyodermatitis–pyostomatitis vegetans is a rare, polymorphous inflammatory disorder of the skin and oral mucosa first described by Hallopeau in 1898. On the skin papules, pustules and reddish brown annular vegetating plaques develop, most frequently in the intertriginous areas. In the mouth, yellowish flat ulcerations arise, typically in the shape of "snail tracks". The association with inflammatory bowel disease is very common. An unusual case with a chronic relapsing course of 2 decades is presented. Gastrointestinal inflammation was absent. Prednisolone in high and medium doses suppressed most lesions. Various attempts with other drugs (dapsone, isotretinoin, azathioprine) to reduce the corticosteroid dose failed. This is the first report of the successful treatment of pyodermatitis–pyostomatitis vegetans with cyclosporin A, which proved to be highly effective in this regard. The unknown aetiopathology of pyodermatitis–pyostomatitis vegetans is discussed. ***Key words: Hallopeau; pathogenesis; inflammatory bowel disease.***

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After a century of scientific dispute, at present pyodermatitis–pyostomatitis vegetans (PPV) is generally considered to be a distinct clinical entity in the spectrum of chronic pustular dermatoses. It is an uncommon inflammatory disease of the skin and/or mucosal membranes with characteristic vegetating lesions. The infiltrate is polymorphic and eosinophils are often present. However, diagnosis is largely based on exclusion criteria. The aetiology and pathogenesis of PPV are unknown. An association of PPV with inflammatory bowel disease is well established.

CASE REPORT

In October 1979, a 32-year-old Caucasian clerk was referred to our department with a 1-week history of pustular orogenital lesions (Fig. 1) and a papulopustular skin eruption affecting his back, intertriginous areas, feet and heels. Systemic therapy with corticosteroids, antibiotics and immunoglobulins caused remarkable relief. One year later, in September 1980, the patient was admitted again presenting with rapidly progressive, erythematous, sharply demarcated, discoid to annular, pustule-fringed plaques, located predominantly on his extremities, axillae and groins (Fig. 2). Antecedent drug intake was denied, and no other trigger factor was detectable. The patient was otherwise healthy, apyrexic and on no medications. His family history was non-contributory. Treatment was similar to that on the previous stay. After 4 weeks the patient was discharged with a grossly healed integument. Since 1991 mild exacerbations with hyperkeratotic plaques occurred on the inguinal skin and could be controlled by local application of triamcinolone ointment.

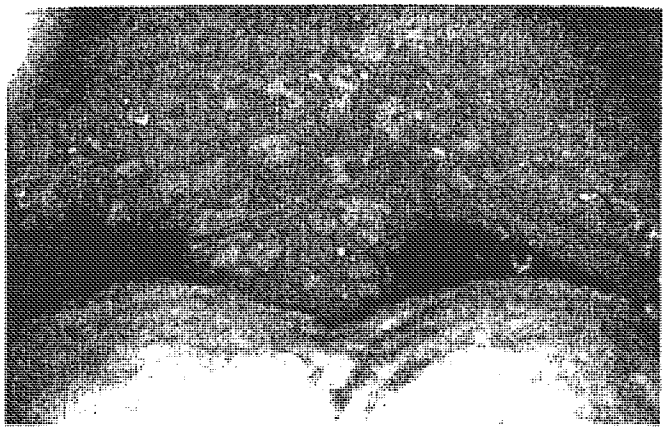

Fig. 1. Confluent pustules on the palate (early stage).

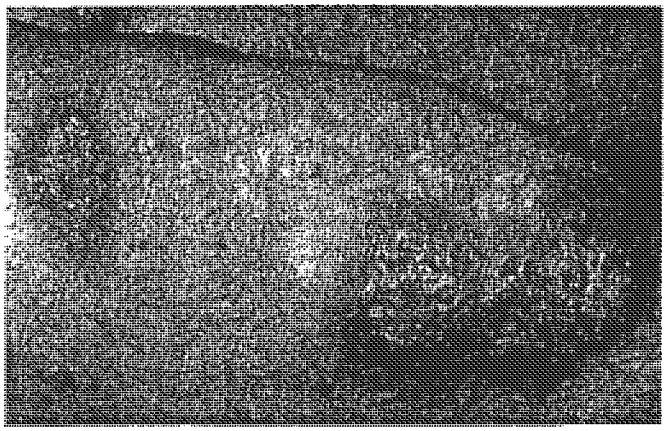

Fig. 2. Late-stage vegetating annular lesions on the legs.

In April 1998 the patient developed a generalized recurrence of erythematous papulopustules involving the scalp, inguinal areas, perianal region and elbows. The enanthema was characterized by friable tiny yellow pustules affecting chiefly the buccal and labial mucosa, soft and hard palate, as well as the tip of the tongue. The patient was hospitalized with the working diagnosis of PPV. Systemic administration of corticosteroids, starting with 80 mg methylprednisolone per day and slowly titrated to a maintenance dosage of 8 mg, once more succeeded in complete remission. The patient was discharged in May 1998. After an acute re-exacerbation in the groins in August 1998 a trial with dapsone (100 mg/day) was undertaken but failed because of a new bout in October 1998. In the groins and axillae large indurated plaques were present with draining sinuses. There were no oral lesions on this occasion. The patient was readmitted and dapsone was discontinued. In addition to oral prednisolone (20 mg/day) the patient received antibiotic infusions with ceftriaxon and ampicillin/sulbactam

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because of bacterially colonized skin lesions. A treatment with isotretinoin 50 mg/day was started while prednisolone was tapered off. After 3 weeks the draining plaques in the intertriginous areas dried up. The patient was discharged in November 1998 with marked regression of his skin lesions.

Five months later isotretinoin was discontinued because of a remarkable elevation in the liver enzymes. Within the next month the predilection sites at his groins and buttocks displayed new painful lesions. Oral prednisolone (20–30 mg/day) was required again. In September 1999 a new combination therapy with azathioprine (50–100 mg/day) was initiated to lower the corticosteroid maintenance dose. Within 7 weeks of drug intake the vegetations had not improved considerably. The patient had to stop taking azathioprine because of transient diarrhoea. He continued on a monovalent medication of 20–30 mg prednisolone on alternate days. Several attempts to taper the dose below 20 mg/day led to relapse. In December 1999 the patient agreed to a combination with cyclosporin A (4.5 mg/kg per day) in an oral formulation. All skin lesions healed within 3 months. The dose of oral prednisolone was lowered to 7.5 mg/day and cyclosporin A was reduced to 2.5 mg/kg per day. He continues to receive these doses.

Investigations

Haematological investigations revealed leukocytosis (median white blood cell count 13×10^9 cells/l) and a differential count with lymphopenia (20%). Further routine biochemical investigations (haematology, serum chemistry) were normal on several occasions. A urinalysis failed to indicate increased excretion of halogens. The three main classes of immunoglobulins (IgG, IgM, IgA) were slightly raised. Autoantibodies, antinuclear antibodies and antineutrophil cytoplasmic antibodies were within normal limits. Infection markers [TPHA, Tine test, hepatitis screen, anti-herpes simplex virus (HSV)-1/2, anti-human immunodeficiency virus (HIV)-1/2, anti-cytomegalovirus (CMV)] were negative. The relative distribution of all lymphocyte subpopulations revealed no evident aberrations. Human leukocyte antigen (HLA)-B27 and -B5 were negative.

Various lesional skin swabs for microbiology produced bacterial growth for *Staphylococcus aureus*, *Klebsiella*, *Bacteroides coli*, α-haemolytic *Streptococci* and *Enterococcus* species. Controls were negative after treatment.

X-ray photographs of the chest, paranasal sinus and teeth were always within normal limits. Screening for gastrointestinal involvement was negative using abdominal ultrasonography and laboratory (faecal occult blood tests, stool cultures), endoscopic (colonoscopy) or radiological (Sellink) techniques as well as magnetic resonance imaging scans.

The main histopathological findings of repeated skin biopsies comprised a hyperparakeratotic and acanthotic epidermis with intraepidermal abscess formation. A dense perivascular cell infiltrate was composed of histiocytes, a few plasma cells and eosinophils. Stains for bacteria and fungi were negative. Repeated direct and indirect immunofluorescence studies were consistently negative for intercellular or basement membrane zone staining. In addition, immunoblotting did not show any characteristic bands.

DISCUSSION

The diagnosis of a chronic vegetating pustular dermatosis must be considered as a general term which needs further differentiation. Hallopeau (1) was the first to enlarge the old terminology, coining the term "pyodermite vegetante". PPV may occur in any age group and shows a gender predilection for males. The oral cavity is not always affected in this disease, although it can be the sole manifestation (2). Pyostomatitis may precede, coincide with or follow pyodermatitis. The skin lesions in PPV, seen in approximately 40 patients to date, have repeatedly been reviewed, preferentially in tabular form (3–9). The typical manifestation sites in PPV are the oral mucosa and the intertriginous areas of the skin. As in the present case, PPV may exhibit extremely variable clinical manifestations within the same patient. Skin lesions characteristically start as erythematous papulopustules and extend to annular vegetating plaques. The typical "snail track" appearance of oral lesions refers to eroded lesions that coalesce. The histological features are summarized in Table I. The association with inflammatory bowel disease (IBD) occurs in about 70% of cases (10). A link between PPV and liver dysfunction or primary sclerosing cholangitis has also been supposed (4, 11). Immunofluorescent investigations are obligatory to differentiate PPV from pemphigus vegetans (12, 13). In the present case neither immunofluorescent investigations nor immunoblotting (14) were positive, which separates it clearly from the bullous autoimmune diseases. Important differential diagnoses were carefully excluded (Table II).

The aetiopathogenesis of PPV is unknown. The observation that lesions tend to develop in the intertriginous areas where moisture and friction promote microbial growth suggests an idiosyncratic reaction to microbial agents. Potentially toxin-producing bacteria were cultured from the vegetating lesions in this patient. It seems incongruous, however, that bacterial and fungal cultures can be negative (5, 10). Consequently, the idea of non-causative infections superimposed on the underlying disease has been favoured (15). Nevertheless, it is possible that microbes are relevant to disease progression in patients with PPV. Hidden immune dysfunction might play a synergistic role (16). Infectious agents and immunological mechanisms have also been discussed as putative causes of IBD (17). PPV serves the function as a marker for associated IBD since the gastrointestinal disease can be asymptomatic for a certain period (5, 9). Pathogenetically, there are still knowledge gaps related to how exactly components of the immune system regulate functions of spatially separated tissues (18). Some authors envisage a model in which reactive cutaneous lesions in patients with IBD may be immunologically mediated via cross-antigenicity between the skin and the gut (19) or via epitope spreading (20). It is noteworthy that few patients with pyostomatitis vegetans and ulcerative colitis showed complete remission of the oral lesions immediately after a total colec-

Table I. *Histological features of pyodermatitis–pyostomatitis lesions*

Microabscesses with neutrophils and eosinophils within the epidermis and dermal papillae/upper dermis
Suprabasilar clefts with no or little cell dissociation/acantholysis
Acanthosis/epidermal hyperplasia
Focal ulceration
Papillomatosis
Perivascular and diffuse inflammation with lymphocytes, neutrophils and eosinophils
No bullae, vasculitis or granulomas

Table II. *Differential diagnoses of pyodermatitis–pyostomatitis vegetans*

Pemphigus vegetans
Acne conglobata
Atypical pustular granulomatous drug reaction
Behcet's disease
Syphilis (late stages)
Tuberculosis cutis verrucosa
Deep fungal infections (e.g. blastomycosis)
Bromoderma/iododerma

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tomy (9, 21). The question arises as to whether in patients without IBD, including the present case, enteric bacteria and/or hidden abnormalities in the immunologically competent colonic mucosa could have functioned as a triggering factor for subsequent inflammatory reactions (or reactivation of memory cells) in the skin or oral mucosa.

PPV is often treated with an empirical multimodal regimen. Topical and systemic therapy with corticosteroids is the approach of choice (5, 6, 9, 10). Dapsone and azathioprine are considered as second-line agents (10). In this patient systemic corticosteroids were repeatedly used to gain control of the condition. Dapsone could not prevent new outbreaks. Under oral isotretinoin healing of the verrucous plaques was accelerated. Isotretinoin has been used for the first time in PPV but may be critical in patients with IBD because of its mucocutaneous toxicity. A good response of PPV to systemic corticosteroids has been confirmed in the present case. However, comedication with cyclosporine was needed to remain below the Cushing threshold. There are no previously reported cases of PPV treated with cyclosporine. Joint application with low-dose oral corticosteroids could be of value in recalcitrant cases.

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Influence of topical Cyclosporine A and dissolvent on corneal epithelium permeability of fluorescein

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Abstract. The corneal stroma is the major barrier to penetration for the lipophilic Cyclosporine A (CsA) molecule and prevents the use of the common ophthalmic solvents. At present, corn oil, castor oil and olive oil are the three most commonly used vehicles. The aim of this study was to determine the effect that topically applied CsA dissolved in different oils has on corneal epithelial permeability measured by fluorophotometry. Forty healthy volunteers, with absence of ocular or systemic disease and not receiving topical or systemic drugs were enrolled. Measurements were taken before and 45 min after the instillation of 40 microliters of a 2% aqueous solution of sodium fluorescein without preservatives. Basal corneal permeability and the permeability 24 h after the instillation of 2% CsA-olive oil, olive oil alone, 2% CsA-castor oil, castor oil alone, 2% CsA-corn oil and corn oil alone, were calculated. To prepare the topical 2% CsA, a Sandimmun oral solution (Sandoz, Basel, Switzerland) was employed under sterile conditions. We found that epithelial permeability 24 h after the instillation of any CsA formulations or solvents increased more than 6.62 times ($p < 0.001$). No differences in corneal permeability values were found between any of the CsA formulations and the vehicles. We conclude that oils used to dissolve CsA are mainly responsible for the increased corneal epithelial permeability. No differences were found in the effects of the tested solvents on corneal epithelial permeability.

Introduction

Cyclosporine A (CsA) is a neutral, hydrophobic, cyclic endecapeptide metabolite of the fungus Tolypocladium inflatum gans [1]. Current data suggest that CsA has a selective suppressing action on T lymphocite immuno-mediated reactions. Systemic administration of CsA has been employed to treat corneal graft rejections, uveitis, Grave's ophthalmopathy, myastenia gravis and a wide variety of ocular immune-mediated disorders [2]. However, systemic side effects, such as nephrotoxicity [3], hepatotoxicity [4] and hypertension [5] limit the usefulness of systemic administration. Therefore, topically applied CsA has been used as an alternative in preventing corneal allograft rejection [2, 6–8], vernal keratoconjunctivitis [9], ligneous conjunctivitis [10] and

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autoimmune corneal ulcers [11]. Topical CsA inhibits the afferent antigen-mediated lymphokine release, thus preventing the entry of T lymphocytes into the cornea [1]. No commercial topical CsA formulation is currently available. The lipophilicity and uncharged nature of the molecule interfer with the ocular penetration and prevent the use of the common ophthalmic vehicles [12]. At present, corn oil, castor oil and olive oil are the three most commonly used vehicles [2, 9, 11, 13–15]. The side effects of topical CsA include a burning sensation and an initial transient punctate epitheliopathy [1, 2, 8, 9, 12, 15, 16].

The corneal epithelium is the major barrier for the penetration of lipophobic solutes such as fluorescein. Fluorophotometry has been used to quantify corneal epithelial damage. After topical sodium fluorescein application, modern objective fluorophotometry can detect changes in corneal epithelium permeability accurately [17–20].

Versura *et al.* [15] evaluated the potential toxic effects of topically administered CsA dissolved in olive oil on the cornea in the rabbit eye. They concluded that the vehicle, rather than the drug, was responsible for the superficial epithelial defects developed in the cornea. Benítez del Castillo *et al.* demonstrated by means of fluorophotometry that the olive oil rather than CsA was more responsible for the increase in human corneal epithelial permeability [21].

This study was conducted to evaluate and to compare the effect in corneal epithelial permeability of different oils currently used as CsA vehicles.

Subjects, material and methods

Eighty eyes of 40 healthy volunteer (14 males, 26 females) were evaluated. Their mean age was 21.3 ± 6.5 years old (range, 15 to 41 years). They had no medical history of diabetes mellitus, systemic or ocular diseases and contact lens wearing. They were not taking systemic or topical drugs. The slit-lamp findings were unremarkable and the tear film break-up time was higher than 15 seconds in all cases. Schrimer's test without anesthetic values were higher than 15 mm in 5 min and rose bengal as well as fluorescein staining of the ocular surface were negative.

Approval for the study was obtained from the Ethics Committee of Complutense University and all patients gave informed consent prior to enrollment.

The fluorophotometer used in this study was the FM-2 Fluorotron Master (Coherent Radiation, USA). Measurements were performed 5 days after the slit-lamp examination with fluorescein staining. After measuring the autofluorescence of the tear film and the corneal stroma (C0), 40 microliters of 2%

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Table 1. Number of eyes with epithelial keratitis 30 min after the instillation of the different drugs

CsA-olive	CsA-castor	CsA-corn	Olive	Castor	Corn
64	59	67	60	62	61

aqueous solution of sodium fluorescein with no preservatives was instilled into the lower conjunctival sac. The fluorescence of the corneal stroma was measured 45 min later (C45), when no fluorescein remained in the tear film. Tear samples were collected from the temporal lid margin using 5 microliters micropipettes. Forty five minutes after the instillation no fluorescence remained in the tear film. Therefore, the increase in corneal stroma fluorescence was directly related to the corneal permeability. The corneal epithelial permeability was calculated by substracting the stromal autofluorescence value from the value obtained at 45 min (C45-C0) [17, 18]. Topical 2% CsA was prepared in the hospital pharmacy under sterile conditions using commercially available oral CsA (Sandimmun, Sandoz, Basle, Switzerland) diluted with olive oil, castor oil and corn oil respectively.

The oral solution of CsA contains 100 mg/ml of a mixture of 30 parts peglicol-5-oleate, an emulsifier, 60 parts olive oil and 10 parts ethanol [1]. The preparation requires alcohol evaporation in a ventilation hood for a 24 h period. Radioimmunoassay (RIA) results confirmed a 20 mg/ml CsA concentration.

Epithelial corneal permeability was calculated 24 h after the application of one drop of the following solutions: 2% CsA-olive oil, olive oil, 2% CsA-castor oil, castor oil, 2% CsA-corn oil and corn oil. A one-week interval was established between the application of a new solution. The tear film analysis (chromatographic and RIA) performed 24 h after the instillations, showed absence of oil or CsA.

All fluorophotometrics measurements were carried out between 3 p.m. and 5 p.m. After drug topical application, symptoms (itching and foreign body sensation) and signs (conjunctival hyperemia) were recorded. Slit-lamp examinations were performed at 30 min and 24 h after drug application.

For statistical analysis, Student's t-test for paired data was used. We consider $p < 0.05$ to be statistically significant. The value of the corneal permeability was the average of both eyes [22].

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Table 2. Corneal permeability values (C45-C0) (ng/ml) baseline and after the application of the six different drugs (Mean ± SD)

	Permeability (ng/ml)
Baseline	34.4 ± 23.5
CsA-olive	251.1 ± 119.6
CsA-castor	244.2 ± 105.9
CsA-corn	258.1 ± 97.4
Olive oil	233.9 ± 106.3
Castor oil	240.8 ± 111.4
Corn oil	244.3 ± 99.3

Results

After the instillation of the six products tested, all subjects complained of itching, tearing or foreign body sensation for approximately one hour. No epithelial keratitis on slit-lamp examination was detected at 24 h after the instillation. However, at 30 min after the instillation, 64 eyes treated with CsA-olive oil, 59 eyes treated with CsA-castor oil, 67 eyes treated with CsA-corn oil, 60 eyes treated with olive oil, 62 eyes treated with castor oil and 61 eyes treated with corn oil alone developed punctate keratopathy ($p > 0.05$) (Table 1). Thirty minutes after the instillation of any drug, all subjects showed mild conjunctival injection. Tear production (Schrirmer's test) measured 25 h after drug application, showed no significant increase.

The results of the right eye baseline permeability values (35.9 ± 22.6 ng/ml) were compared with the left eye baseline permeability values (32.9 ± 24.5 ng/ml) and the variability coefficient was calculated. The variability in both eyes was 9.5%. To determine more accurately this assay, the baseline test was repeated one week later in 10 normal volunteers and the variability value was 12%. The high values in corneal permeability standar deviations were due to interindividual differences.

We did not detect an increase in corneal autofluorescence after the instillation of any drug. The corneal permeability values (C45-C0) are shown in Table 2. Comparing with baseline data, we observed a significant increase of epithelial permeability after 24 h of applying CsA-olive oil (7.3-fold increase, $p < 0.001$), CsA-castor-oil (7.1-fold increase, $p < 0.001$), CsA-corn oil (7.5-fold increase, $p < 0.001$), olive oil (6.8-fold increase, $p < 0.001$), castor oil (7-fold increase, $p < 0.001$) and corn oil (7.1-fold increase, $p < 0.001$). No difference in corneal perm formulations and vehicles

Discussion

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difference in corneal permeability values were found between any of the CsA formulations and vehicles.

Discussion

In an attempt to avoid the potentially severe side effects associated with the systemic administration of cyclosporine, investigation has been directed towards local administration of CsA. The epithelial cell layer of the cornea is hydrophobic and easily penetrated by lipid-soluble substances such as CsA [23]. Corneal levels of CsA obtained after topical administration are above the therapeutic range providing a local immunosupresiuve activity capable of preventing unwanted ocular responses such as corneal allograft reactions [24]. Topical CsA presumably inhibits the afferent antigen-mediated lymphokine release; therefore, long-term treatment would be necessary in order to prevent graft rejection [1]. Furthermore, the drug and its solvent should be well tolerated and safe.

Previous clinical studies have not directly shown the toxic effects on the human cornea of topical CsA and/or its solvents. Clinically, topical CsA seems to be well tolerated over the long term without significant local toxic effects. However, following topical administration of CsA an initial transient punctate epitheliopathy and some mild conjunctival injection has occasionally been reported [1, 2, 8, 9, 16].

The morphological substrate of the epithelial diffusion barrier is made up of apically located zonulae occludentes which seal off the epithelial intercellular spaces. Even minor lesions of the cornea surface, too small to be recognized with the slitlamp, may result in an impairment of the corneal epithelial barrier function which can be quantified *in vivo* by means of fluorophotometry [18].

In our study, an increased amount of fluorescein in the corneal stroma was found after the application of CsA-vehicles. Our results suggests that high fluorescein concentrations were mainly due to the vehicles because after applying the oils alone the results were similar as those with CsA-oil.

Furthermore, we found a change in corneal epithelial barrier at 24 h after the instillation of 2% CsA-vehicle due to the solvents. We did not find differences in slit-lamp examination (epithelial keratitis and conjunctival hyperemia) after applying all solutions. The breakdown of the epithelial barrier could improve the corneal penetration of CsA. However, the CsA is a lipophilic substance and penetrates well in epithelium. For that, the breakdown improves the penetration of water-soluble solutions, such as some steroids (phosphate derivatives of prednisolone and dexamethasone). This is important in corneal graft, where the epithelial permeability is affected after

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the surgery and the immunosupresive effect of topical CsA could be partially due to the increased corneal penetration of topical corticosteroids. Further studies with a long-term treatment are needed, because the CsA induced epithelial defects reported in the literature are transient ones (2–9 months) [1, 2, 16].

The epithelial damage caused by CsA-vehicle could partially cause an increase in lacrimation in tear deficiency. In this study we did not detect an increase in Schrimer's test after applying the drugs but our patients had normal baseline tear production.

By using transmission and scanning electron microscopy in the non-grafted rabbit eye, Versura *et al.* have shown that it was the olive oil rather than CsA which was responsible for the surface epithelial defects developed on the cornea. In their study, six rabbits received topically applied 1% CsA dissolved in olive oil in the right eye and olive oil in the left control eye (three drops five times a day for 4 weeks). Both, CsA-treated and control eyes showed evidence of corneal epithelial defects which were detected by rose bengal staining and slit-lamp examination. By scanning electron microscopy, the superficial cells appeared to exfoliate as sheets of cells and by transmission electron microscopy, the plasma membrane of the basal epithelial cells appeared to be interrupted in some points. They also found an electron-dense material, which could be oil, at the level of the intercellular spaces or within the cytoplasm of the epithelial cells 24 h after the last dose was administered [15]. This study, even though it was performed in the rabbit eye, could explain the mechanism and the increase of epithelial permeability found by our group in the human eye.

In conclusion, we found that oils employed to dissolve topical CsA are the main responsible for the breakdown of corneal epithelial permeability. We did not find differences in the barrier damage caused by the solvents tested (olive oil, castor oil and corn oil). We suggest that a less toxic vehicle to administer topical CsA may be needed.

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Influence of vehicle and anterior chamber protein concentration on cyclosporine penetration through the isolated rabbit cornea

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ABSTRACT

The transcorneal penetration of cyclosporine A has been determined from each of three vehicles across isolated cornea into simulated aqueous humor containing either 50 mg % protein (0.5 mg/ml; as found in a normal eye) or 5000 mg % protein (50 mg/ml; as found in an inflamed eye). Cyclosporine entered the corneal epithelium and stroma/endothelium as well as passed through the cornea from an alpha cyclodextrin vehicle. Entry into the epithelium and stroma/endothelium occurred from an ointment vehicle with limited detectable anterior chamber penetration using 50 mg % protein solution in the anterior chamber. From corn oil vehicle, cyclosporine penetrated across the cornea with a permeability equal to that of alpha cyclodextrin vehicle. The concentration of cyclosporine in both corn oil and ointment vehicles is 8 times greater than that in alpha cyclodextrin vehicle resulting in a flux from corn oil vehicle about 7 or 8 times greater than that seen after alpha cyclodextrin vehicle. The amounts retained in the cornea, however, were relatively low after corn oil compared to cyclodextrin. The penetration of cyclosporine from either the cyclodextrin vehicle or ointment was at least doubled in the presence of 5000 mg % protein in the simulated aqueous humor relative to that seen in 50 mg % protein. This data indicates that the (presumed) absorption and binding of drug by the excess protein in the simulated aqueous humor may have removed free cyclosporine from the solution and sustained a high concentration gradient of free solute across the cornea. This occurs despite the proven binding of cyclosporine to the tubing leading from the corneal chamber to the collection vial, although the influence of albumin upon this binding is relatively small. The vehicle governs the release and penetration of cyclosporine to and through the cornea, with corn oil and cyclodextrin giving the same corneal permeability to cyclosporine but since corn oil can contain a greater drug concentration it provides a greater flux of the drug.

INTRODUCTION

The immunosuppressive drug cyclosporine A (CsA) has been shown to be efficacious in alleviating complications associated with inflammatory ocular diseases (1–8) as well as corneal transplantation (9–13). The ocular penetration of CsA has been examined under several experimental conditions (14–33) and conflicting results have been obtained. The topical administration of CsA in olive oil has been reported to cause high corneal and conjunctival concentrations of the drug with none appearing in intraocular structures although in inflamed eyes high CsA levels were found within the anterior segment (1). The data stands in contradistinction to other reported values (20,21) although being substantiated by another report (16). The increased penetration of CsA after a significant inflammation of the eye (15) was attributed to the edematous nature of the cornea and disruption of the epithelium.

The present in vitro study was made to determine whether or not CsA passes through the isolated cornea under two types of conditions. One variable was the vehicle, either an ointment, corn oil, or a cyclodextrin A-based solution (24–27). We compared, therefore, a low capacity (cyclodextrin) vehicle with a high capacity (ointment) vehicle that have different characteristics towards this lipophilic drug. The second variable was the protein content of the artificial aqueous humor, ranging from a

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simulated normal aqueous protein content of 50 mg % (28,29) to that of severe inflammation, up to 5000 mg % (28-31).

MATERIALS AND METHODS

Adult albino rabbits, weighing 2 to 3 kg, were killed with an intravenous overdose of sodium pentobarbital (5 ml of a 65 mg/ml solution) given via a marginal ear vein. Eyes were immediately removed and prepared for mounting in the specular microscope with intact epithelia (33-34). The endothelial surface was perfused at 3 ml/hour with Ringer bicarbonate solution (35) using a Harvard infusion pump (Harvard Instruments, Boston, MA) and the effluent collected at 30 min intervals for duplicate sampling and subsequent counting. The epithelial surface received CsA in either ointment, corn oil, or a cyclodextrin solution. Radioactive ^{3}H CsA (Amersham Corporation, Arlington Heights, IL; prepared by sodium boro[^{3}H]hydride reduction) was added to an ointment (petrolatum base) containing 0.3% CsA, corn oil containing 0.2% CsA, or an alpha cyclodextrin solution at 40 mg/ml containing 0.025% CsA, to give a count rate that averaged about 2.2 x 10^{6} cpm per 100 µl or 100 mg vehicle. This combination of alpha cyclodextrin and cyclosporine concentrations was chosen on the basis of prior data on corneal toxicity and drug penetration (26,27); the combination also approaches the maximal CsA concentration that can be solubilized in this concentration of alpha cyclodextrin. As accurately as possible the same quantity of CsA vehicle was applied to the corneal surface. The solution volumes were 350 µl and an equivalent amount of the ointment was spread over the corneal surface.

The ointment or solution was placed on the epithelial surface for the duration of the experiment. The penetration of ^{3}H CsA through the cornea was determined by sampling and counting the effluent from the endothelial chamber over a 3 to 5 hour period. The solution bathing the endothelium contained either 50 mg % (0.5 mg/ml) or 5000 mg % (50 mg/ml) bovine serum albumin. At the end of the experiment the epithelial surface was washed well with non-radioactive saline and the epithelium harvested by scraping using a Gill corneal knife. A stromal button was trephined from the cornea and both epithelial and stromal samples were placed in 100 µl volumes of deionized water overnight. Duplicate samples of the deionized water were taken, after at least 24 hours of CsA washout, and counted to provide values for tissue uptake of cyclosporine. Preliminary studies showed that the recovery of CsA from stromal buttons solubilized in Protosol® versus extraction in deionized water was almost identical. Stromal buttons (10 per extraction process) received equal volumes of ^{3}H-CsA and were allowed to take up 10 µl applied to the de-epithelialized surface. When uptake was complete extraction occurred into 1 ml of either water or Protosol® for 24 hours. The recovery of CsA from Protosol® was between 5 and 11% greater than that from deionized water when compared with each other. The different percentage values corresponded to Protosol® samples that were neutralized with HCl prior to mixing with ScintiVerse (5%) relative to non-neutralized samples (11%), respectively. The overall recovery rates for these processes were 11 and 12% of the added CsA for water and Protosol®, respectively. The extraction procedure for CsA from epithelial cells was considered complete because of the disruption of cells when immersed in deionized water that would release all of the cell contents regardless of the nature of the drug.

The possibility was raised of cyclosporine binding to polyethylene tubing (36,37), used as an effluent from the chamber to the collection vial, or of the albumin preventing cyclosporine binding to the

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Table 1.

Rabbit corneal cyclosporine flux, and corneal content, from ointment, corn oil or alpha cyclodextrin vehicle into low or high protein solutions.

Vehicle	CsA Concentration (μg/ml)	Endothelial Bathing Bathing Solution Protein Concentration	Permeability cm/hr x 10^{-3}	Flux (μg x 10^{-4}/ cm^2/hr)	Content (μg/mg wet weight) Epi	Str/Endo
Alpha-cyclo-dextrin	0.25	50 mg %[a]	6.6	18.3±0.8	0.30±0.05[g](5)	0.13±0.01
		5000 mg %[b]	10.0	25.0±2.6[c](7)	0.12±0.02[g](7)	0.13±0.02(7)
Ointment	2.0	50 mg %	0.4	8.4±2.9[e]	0.13±0.03[c,g](5)	0.14±0.04(5)
		5000 mg %	1.7	35.0±8.1[d]	0.14±0.02[g]	0.10±0.02
Corn oil	2.0	50 mg %	6.9	137.1±32.9[c]	0.04±0.01[c,g](5)	0.026±0.01(5)

Values are the mean ± SEM of 6 experiments with each experimental variable unless indicated otherwise in parentheses. CsA concentration = concentration in application vehicles; Flux = permeability x concentration; Epi = epithelium alone; Str/Endo = stroma plus endothelium. [c], significantly different from values with cyclodextrin obtained with 50 mg % protein on endothelial surface, ($P < 0.02$); [g], significantly different from cyclodextrin epithelium at same simulated aqueous protein concentration, ($P < 0.01$); [f], significantly different from stroma/endo, ($P < 0.002$). [a], 0.5 mg/ml; [b], 50 mg/ml; [e], calculated based upon a count rate only 1.2 ± 0.3 cpm greater than background of 30.5 cpm; [d], calculated from a count rate of 7.9 ± 0.6 cpm greater than background of 30.5 cpm.

tubing (37), and thus influencing the count rate of the effluent. To investigate this possibility experiments were made in which solutions containing ^{3}H-CsA together with either 50 mg % or 5000 mg % albumin were perfused directly through tubing of the same length as the effluent tubing from the corneal chamber to the collection vial. Solutions were collected at 20 min intervals and sampled as described above for the experimental protocol. Each solution (50 or 5000 mg %) was made from the same stock ^{3}H-CsA so that the initial cpm was the same. The count rate of this hot solution was of the order of 3000 cpm (approximately 1 ng/ml) and non-labeled CsA was not added. This represented a maximal condition for the determination of binding by the tubing at a CsA concentration close to that seen under experimental conditions.

The principles expressed in the Declaration of Helsinki and The Guiding Principles in the Care and Use of Animals (DHEW Publication, NIH 86-23) were strictly adhered to in the performance of the reported studies.

RESULTS

The values for corneal permeability and flux of CsA from the vehicles and into either of the two endothelial bathing solutions, as well as epithelial and stromal CsA concentrations, are given in Table 1.

The transcorneal flux of CsA is greatest from the corn oil vehicle, less from the alpha cyclodextrin vehicle and even lower from ointment into a 50 mg % simulated aqueous humor (Table 1). This occurred despite the similarity in permeability of CsA from the corn oil and cyclodextrin vehicles. The flux values, of course, are a function of the drug concentration as well as permeability, thus the 2 μg/ml concentration of the corn oil and ointment vehicles is

a significant determinant in the calculation of flux.

The values obtained for CsA penetration from alpha cyclodextrin or ointment were dependent upon the protein concentration of the simulated aqueous humor. The flux of CsA into the 5000 mg % protein concentration solution was at least double compared to that with 50 mg % protein in the simulated aqueous. The ointment data must be put into perspective, however, since only a low count rate of the aqueous effluent from ointment vehicle was observed. This value increased from 1.2 ± 0.3 cpm above a background of 30.5 cpm for the 50 mg % solution (n = 15) to 7.9 ± 0.6 cpm above the same background count rate for the 5000 mg % solution (n = 18) (these are uncorrected numbers; corrections were applied for CsA binding to polyethylene tubing [see below]). While these numbers are very low they are statistically different from zero (P<0.03 and <0.01, by Cochran's t-test, respectively) and thus probably represent real penetration of CsA rather than being background variation or noise in the count rate. The flux values are calculated from these low numbers with the associated high error in counting. That flux values can be calculated reflects in a large part the high concentration of CsA in this vehicle (Table 1). If the concentrations were significantly lower so would the calculated flux be proportionately reduced. Nevertheless, the values indicate marginal penetration through the cornea from a concentration as used in these studies.

The greatest amount of CsA was found in the epithelium from the alpha cyclodextrin vehicle with about half that value found after ointment and almost one eighth that value after corn oil vehicle (Table 1). For the rest of the cornea (stroma/endothelium) the values tend to be low per unit weight, probably reflecting the high lipophilicity of the drug. The values shown in Table 1 reflect the recovery rate of 11% found in the extraction studies described above. Only this small percentage recovery occurred regardless of the extraction vehicle (water or Protosol®). The stroma/endothelium contains more CsA on a per total tissue basis, and for most vehicles reflects an even distribution except for the alpha cyclodextrin vehicle where the proportion of drug in the epithelium is greater than for the other vehicles. All reported values were corrected for the recovery rate found in either extraction vehicle.

Perfusion of tubing, equal in length to the effluent tubing from the chamber to the collection vial, indicated that the presence of 5000 mg % albumin prevented some cyclosporine binding to the tubing. Perfusion of tubing in the presence of 50 mg % albumin allowed an average of only 38±2% of the entering CsA to emerge into the collection vial. In the presence of 5000 mg % albumin, however, the fraction of recovered CsA was 84±2% of the input solution. These percentages were found to be quite variable between each of the 20 minute collection periods over a total of 3 hours, as well as from tubing to tubing. The data in Table 1 are corrected for the average change in cpm of the effluent fluid, depending upon the protein content of the perfusion solutions. All data are thus corrected for binding either to albumin and tubing or tubing alone.

DISCUSSION

Several authors have reported that the topical application of cyclosporine in olive oil or castor oil resulted in either no intraocular penetration or a penetration of less than therapeutic levels of CsA into the anterior segment (14-16,18,19). Other authors (20,21), however, have determined that CsA penetrated into the anterior chamber, and other ocular tissues, to therapeutic levels from topically applied solutions. The present studies, albeit in a model in which the surface kinetics of drug application differ substantially from those found in

vivo, were designed to examine whether CsA could penetrate the cornea alone. In the present studies CsA at high concentrations was present for the duration of the experiment, a far greater exposure than any topical drop or ointment administration would provide (38). Furthermore, only the corneal route was tested in this model system to the exclusion of other pathways for drug penetration (e.g., transscleral penetration) into the simulated aqueous humor.

Preliminary studies showed that the permeability of CsA was constant for considerable times (up to 4½ hours) once a steady-state was achieved (40 to 45 minutes after initiation of perfusion). After this time the permeability rapidly increased. We interpreted this change as indicative of a breakdown of the epithelium caused by constant 4 to 5 hour exposure of this membrane to the vehicles. In all subsequent studies that were reported here, the permeability and tissue content of cyclosporine were determined only up to a time of 2½ to 3 hours after achievement of steady state, thus the status of the epithelium was intact.

The binding of cyclosporine by the effluent tubing that ran from the chamber to the collection vial was used to correct the permeability and flux data depending upon the albumin concentration. Given the large difference in protein concentration between the solutions (50 mg % vs. 5000 mg %) the effect of albumin on CsA binding by the tubing was relatively small. The value was only of the order of a 16% increase in cpm exiting the effluent tubing at the higher albumin concentration. This reflected only a small change in CsA passage through the tubing and did not influence conclusions regarding CsA entrance into simulated aqueous humor of different protein concentrations. CsA is known to bind to proteins and this binding, as well as that to plastic, is temperature-dependent with increasing binding at higher temperatures (37). This temperature-dependence may have contributed to the variation in the values for exiting CsA as the temperature of the exit tubing became warmer during the time course of the experiment.

The present data clearly indicate that penetration of CsA into the epithelium and the simulated anterior chamber occurred from the cyclodextrin, ointment and corn oil vehicles. Indeed, the flux of CsA from corn oil was considerably larger than that from cyclodextrin although the permeability was approximately the same (flux = permeability x concentration). This was due to the higher drug concentration (0.2%; 13,000 cpm/μg CsA) in corn oil vehicle relative to the 0.025% (56,000 cpm/μg CsA) concentration in the cyclodextrin vehicle. Transcorneal drug penetration from the ointment vehicle was very low (Table 1) with a permeability (into 50 mg % protein solution) about half that of the other two vehicles. Indeed, its determination approached the limits of detection for this assay (1.2 cpm above a 30.5 cpm background for the 50 mg % solution and 7.9 cpm above background for the 5000 mg % solution).

CsA was found in the corneal epithelium after exposure to ointment in amounts close to those found after exposure of the cornea to cyclodextrin, whereas the epithelial and stromal/ endothelial values after corn oil vehicle were low (Table 1). For epithelium, count rates after cyclodextrin vehicle were about 58,500 cpm (but this does not translate into much greater CsA content because of the low CsA concentration in this vehicle), whereas for ointment and corn oil values of 2,700 and 3,000 cpm were found, respectively. The discrepancy between these count rates and the values shown in Table 1 is accounted for by the difference in count rate of the cyclodextrin and corn oil vehicles (roughly 3.8×10^6 cpm/100 μl) relative to that of the ointment vehicle

(about 1.4 x 10^6 cpm/100 mg).

The transcorneal flux of CsA from corn oil and alpha cyclodextrin vehicles appears to be in proportion to the respective epithelial content of drug. The greater flux noted in the presence of 5000 mg % protein in the simulated aqueous humor is reflected in a reduced epithelial (but not endothelial/stroma) drug content; this may reflect enhanced passage through, rather than retention by, the epithelium. This is a purely passive process in the same vehicle (or at least vehicles that have the same effect on epithelial absorption and transepithelial drug movement), thus flux could be increased without alteration in the epithelial content (Table 1). The high concentration of albumin could be increasing epithelial permeability as well as the capacity of the epithelium to bind CsA. With corn oil vehicle the flux is very high relative to that from other vehicles yet the epithelial concentration is low. This may be due to an effect of the oil directly upon the epithelium, such as an increase in CsA penetration through the epithelium, that would result in a high flux with less epithelial retention of drug. This process is hypothesized to reflect a change in behavior of the tissue (absorption and transcellular passage of drug through the epithelium) to the vehicle. The vehicle or one of its ingredients may apparently influence both the absorption as well as passage of CsA by the corneal epithelium. Various ingredients in vehicles, such as benzalkonium chloride and vehicle viscosity enhancers, have been shown to directly effect drug penetration through the cornea (38).

One possible explanation for the differences in epithelial CsA content could be the retention of adherent vehicle plus drug on the epithelial surface even after the washing procedure. On a priori assumptions concerning viscosity and other physicochemical characteristics one would rank the vehicles from ointment > corn oil > alpha cyclodextrin in order of adherence properties or difficulty in removal by rinsing. The data in Table 1, however, indicates that the epithelial content follows the order: alpha cyclodextrin > ointment > corn oil. This strongly suggests that adherence of vehicle with drug on the epithelial surface does not contribute to the measurement of epithelial CsA content.

In addition to the influence of vehicle on drug penetration it was of interest to determine whether high concentrations of protein on the endothelial surface would influence the rate of CsA penetration. The latter studies were of importance because of the measured penetration of CsA from topical drops into eyes with significant inflammation. Drug penetration under those conditions had been ascribed to disturbance of the epithelium (15). In the present model one of the solutions bathing the endothelium had a high protein concentration, but the cornea was not inflamed and the epithelium was intact and normal. By eliminating the variable of inflammatory epithelial alterations as a contributing factor to any change in permeability, the data demonstrates that aqueous protein concentrations alone can influence the passage of CsA across the cornea. Any increase in CsA penetration in the inflamed eye must, therefore, have been in part due to the effects of the effect of aqueous protein concentration. The effect of (presumed) binding of CsA by aqueous protein which would thereby sustain a high concentration gradient of free CsA across the cornea must play a role. As suggested from other studies (15), however, a contribution of the inflammatory process per se on the epithelium cannot be eliminated. The highest protein concentration is, however, less than 1 mM and could not contribute to any CsA penetration by such routes as bulk flow.

The present study tested only the transcorneal route of CsA penetration. Most lipophilic molecules,

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to which the cell layers offer little or no resistance, find the corneal stroma to be the major barrier to corneal penetration (39,40). Thus, the low rate of drug passage across the cornea, despite the accumulation of large amounts of CsA in the epithelium, is not surprising. For example, benzalkonium chloride is accumulated to a large degree by the epithelium but none penetrates into the anterior chamber of the adult rabbit eye (41,42). Similarly, the poor penetration of CsA across the cornea alone must be viewed from the *in vivo* perspective in that non-corneal routes of penetration may occur in the living eye that would allow intraocular CsA penetration (39, 43,44). This would be compatible with data indicating CsA penetration from topically applied vehicles, yet these vehicles alone may *per se* influence drug penetration.

The results obtained in this study illustrate that the ocular penetration of CsA across the cornea is vehicle-dependent. More CsA was delivered through the cornea with a cyclodextrin and corn oil vehicle relative to an ointment, despite epithelial penetration with all vehicles. Overall, the corn oil vehicle provided greater trans-corneal drug flux because it contained a greater concentration than the cyclodextrin vehicle. The latter offers good drug penetration but a limited reservoir due to the ability to only hold a low quantity of this drug. This finding is of importance in the development of vehicles for either the ocular penetration of CsA or the restriction of CsA to the ocular surface.

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DC - B05
DCR - [1] 103243 CL; 90981 CL; 194612 CL
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IC - A61K45/08; A61K38/00; A61K47/06; A61K47/24; A61K9/107; A61P27/02; A61P27/16; A61P37/06
IN - SUZUKI S; TAKEUCHI M; YAGATA H; YAMAZAKI S
LNKA- 2000-148260
M1 - [01] M210 M211 M212 M213 M214 M215 M216 M220 M221 M222 M223 M224 M225 M226 M231 M232 M233 M320 M416 M423 M431 M610 M620 M782 M905 P433 P714 P812 P922 P943 R022; RA00NG-K RA00NG-M RA00NG-T
- [02] F011 F012 F013 F014 F015 F016 F017 F590 H2 H213 H4 H401 H481 H7 H721 H8 J5 J523 L9 L941 L999 M210 M211 M212 M213 M214 M232 M240 M273 M283 M315 M321 M333 M340 M342 M373 M391 M423 M431 M510 M521 M530 M540 M782 M904 M905 P433 P714 P812 P922 P943 R022; RA0135-K RA0135-M RA0135-T
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MC - B02-C01 B04-B01B B05-B01P B10-J02 B12-M03 B14-G02 B14-N01 B14-N03 B14-N17C
PA - (WAKA) WAKAMOTO PHARM CO LTD
PN - JP2000143542 A 20000523 DW200044
PR - JP19980318786 19981110
XIC - A61K-045/08; A61K-038/00; A61K-047/06; A61K-047/24; A61K-009/107; A61P-027/02; A61P-027/16; A61P-037/06
AB - NOVELTY :
Oil-in-water (O/W) emulsion formulation comprises a slightly soluble emulsion, an immuno-suppressive agent, a phospholipid and liquid paraffin.
- ACTIVITY :
Immuno suppressive; ophthalmological; antipsoriatic; antiinflammatory.
No test details for the above activity are given.
- MECHANISM OF ACTION :
None given.
- USE :
The emulsion is useful for treating allograft rejection, bone marrow rejection, psoriasis vulgaris, aplastic anemia and nephrotic syndrome, Behcet's syndrome, herpetic keratitis, chronic karatitis, uveitis and corneal transplantation.
- ADVANTAGE :
High drug efficacy is obtained. The emulsion is safe to use and can be used for various treatment.
- PHARMACEUTICALS :
Preferred Component: The slightly soluble immuno-suppressive agent is cyclosporin.
- ADMINISTRATION :
The emulsion containing 0.001-0.5, preferably 0.01-0.2 w/v, % is

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applied as eyedrops.

- EXAMPLE :

Eyedrop of oil-in-water type emulsion was prepared by adding purified egg yolk lecithin (0.19 w/v %), PCSH hydrogen addition soybean phosphotidyl choline (0.08 w/v %), liquid paraffin (0.03 w/v %), EDTA (0.01 w/v %), benzyl alcohol (0.5 w/v %), glycerol (2.2 w/v %) and water for injection having a pH of 6. The above solution was added to cyclosporin A (0.01 w/v %) and emulsion was prepared.

INW - SUZUKI S; TAKEUCHI M; YAGATA H; YAMAZAKI S

IW - OIL WATER EMULSION FORMULATION TREAT REJECT BONE MARROW COMPRISE SLIGHT SOLUBLE IMMUNO SUPPRESS AGENT PHOSPHOLIPID LIQUID PARAFFIN

IWW - OIL WATER EMULSION FORMULATION TREAT REJECT BONE MARROW COMPRISE SLIGHT SOLUBLE IMMUNO SUPPRESS AGENT PHOSPHOLIPID LIQUID PARAFFIN

NC - 1

NPN - 1

OPD - 1998-11-10

PAW - (WAKA) WAKAMOTO PHARM CO LTD

PD - 2000-05-23

RRL - 63917

TI - Oil-in-water emulsion formulation for treating allograft rejection, bone marrow rejection, comprises slightly soluble emulsion, immuno suppressive agent, phospholipid and liquid paraffin

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CYCLOSPORINE OPHTHALMIC O/W EMULSION: FORMULATION AND EMULSION CHARACTERIZATION.

Shulin Ding*, and Orest Olejnik. Pharmaceutical Sciences, Research and Development, Allergan Inc., Irvine, CA 92612

Objective. To develop a non-oily, water based cyclosporine eye drop for topical ophthalmic human use, and characterize its major physicochemical properties. **Method.** Standard methods were used for pH and osmolality measurements. Globule size was determined using single-particle optical sensing and static light scattering technologies. Rheologic properties were measured using a rheometer. A bench-top high-shear mixer was used for laboratory-scale emulsion preparation. **Results.** A stable, pH-adjusted, oil-in-water emulsion was developed using castor oil as the internal phase to solubilize cyclosporine, polysorbate 80 as the primary emulsifier, and a polyelectrolyte as a stabilizer. The concentration of cyclosporine in the oil globule is formulated at the level of 7.4% w/w, which is below the solubility of the drug in castor oil (9-10% w/w). Studies (solubility, interfacial tension, ζ potential, stability) were conducted to support the use of each major excipient. The majority of globules are submicron in size with a bimodal size distribution profile. The globules bear negative charges with a ζ potential of approximately -50 mv. The emulsion was demonstrated to be stable at room temperature for at least 18 months. **Conclusion.** An oil-in-water emulsion of cyclosporine has been developed for topical ophthalmic human use.

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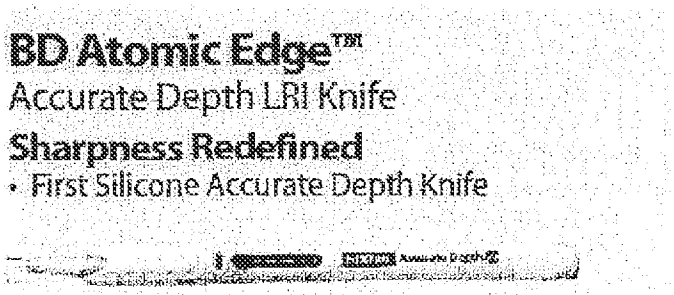
rx perspective

The Economics of Using Restasis

Therapy with cyclosporine 0.05% brings real relief -- and can save doctors and dry eye patients time and money.

By Eric D. Donnenfeld, M.D.



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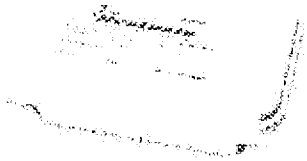
In the past, dry eye patients were often seen as a nuisance -- chronic complainers whom no amount of treatment seemed to satisfy. Despite trying to help these patients with everything in our arsenals (tears, gels, ointments, steroids, plugs, NSAIDS, etc.) nothing provided anything beyond temporary symptomatic relief.



Now, that's all changing, thanks to the availability of Restasis (cyclosporine ophthalmic emulsion 0.05%). (Restasis was approved by the FDA for the treatment of dry eye in late December 2002.) Unique among dry-eye care agents, cyclosporine -- a topical immuno-modulator with anti-inflammatory effects -- attacks the root of the dry eye problem: the underlying inflammation. It doesn't just temporarily alleviate the symptoms, the way artificial tears, lubricants and punctal plugs do; Restasis actually stops the progression of the disease. (If left untreated, dry eye syndrome can cause worsening symptoms, possibly resulting in irreversible damage to the lacrimal gland.)

Thanks to Restasis, there's no longer any reason to view these patients as nuisances, or to think of finding an effective treatment for dry eye disease as problematic.

Economic Benefits

At this point the therapeutic benefits of Restasis are increasingly well known and accepted by the medical community, but -- because of the cost -- there's less consensus about whether it's economically practical to prescribe it.

These concerns, however, fail to take into account many positive economic considerations. As Warren Cross, M.D., senior surgeon and co-researcher at the Bellaire Eye and Laser Center in Houston, noted in his article on this subject in Managed Care Interface in September 2002, "There is a concern about the price of Restasis that is unfounded."

In fact, the positive effect of Restasis on the economics of eye care and on managed care should be huge. Benefits

connected with the use of Restasis include:

Surgical Benefits

Restasis is helping many of my surgical patients achieve better outcomes and higher satisfaction:

- Before approving a patient for LASIK, we perform an extensive dry eye evaluation including a dry eye history, lissamine green corneal and conjunctival staining, and Schirmer testing. I prescribe Restasis for any patient with preoperative dry eye and ocular surface staining. I then allow 1 to 3 months for the dry eye to improve before I perform refractive surgery.
  By preoperatively improving these patients' dry eye problem, I've been able to markedly decrease the incidence of postoperative dry eye.
- My cataract patients with dry eye and visual complaints have noted a dramatic improvement in their visual acuity following Restasis therapy.
- Restasis has also been invaluable for corneal transplant patients who show symptoms of dry eye. Restasis not only treats their symptoms, but helps prevent graft rejection.

Fewer doctor visits. Patients save money on co-pays because they no longer need to visit their eyecare professional again and again in a continuing search for relief from dry eye symptoms.

In Cross' retrospective study of 181 patients using cyclosporine for dry eye, 1,800 office visits were recorded in the 2 years before enrollment in the cyclosporine trials. These visits were specifically motivated by dry eye complaints ("for cause" visits).

After these same patients began using cyclosporine, the "for cause" visits dropped from 1,800 to 38 visits during the following 2 years. In contrast, data from previous non-cyclosporine 0.05% clinical trials involving dry eye patients indicate that 75% of the subjects receiving an active agent felt the same -- or worse -- after the study than in the 12 months preceding it.

Because Cross' study was conducted before Restasis was launched, new studies are needed to confirm the results. However, I've already noticed the same effect on "for cause" visits with my patients. Thanks to the stabilization of their ocular surface, several of my Restasis patients have decreased their office visits from every month or two to twice a year. (Their visits were originally scheduled 3 months apart, but they'd often show up for unscheduled emergency visits.)

In addition, their office visits are now shorter and they leave much happier than they did before they were treated with Restasis.

Fewer related medical costs. In his article, Cross points out: "From an economic perspective dry eye disease is often viewed as having relatively few significant medical costs. However, when use of ancillary medications and more invasive medical treatments (punctal plugs or punctal occlusion) are considered, these costs may rise significantly . . . . Patients with dry eye disease are at greater risk for developing other ophthalmic problems requiring additional medical treatment . . . . Cyclosporine A appears to have potential benefits for health care providers and payers in terms of health care-resource utilization."

Indeed, those benefiting from this economic impact include insurance companies, HMOs, POSs and PPOs. As mentioned earlier, in these trials, insurers ended up paying for only 38 visits after Restasis treatment, instead of 1,800. Also, the large number of indirectly related prescriptions the plans had to pay for decreased dramatically.

Insurance coverage. Insurance coverage, which most of these patients have, makes the cost of Restasis significantly less than retail. My patients' insurers generally charge $30 or less as co-pay for a 1-month supply -- about one dollar a day for a revolutionary new medication.

Less need for artificial tears. Patients won't need to continue buying palliative artificial tears that don't treat the cause of the problem (and aren't covered by insurance). Although artificial tears (e.g., Refresh) can and should be used intermittently with Restasis for additional symptom relief, the FDA clinical studies found that use of supplemental tears gradually wanes as Restasis begins to relieve dry eye symptoms. Many patients eventually stop using them altogether.

Fewer lost work days. Dry eye disease often affects a person's ability to work, so this also factors into the pharmacoeconomic equation. Not only is the condition itself a major source of lost work days, but so are its side-effects and psychological impact. I suspect this new therapy will cause a major reduction in the number of "lost work hours" attributable to dry eye disease.

In addition to these economic considerations, I've found that patients can extend the use of one vial of medication, thereby reducing their costs even further. The packaging says to use two vials a day, but I tell my patients to use one vial a day --

one drop at night and then another in the morning.

I have them store the open vial in the refrigerator (in something that will keep it upright, such as a mug). By using one vial a day, the patient effectively cuts the retail price in half. (I don't advise my patients to use the same vial for longer periods of time because of the risk of contamination.)

At Last: Results

Restasis is good news for our dry eye patients. We now can offer them a solution that will increase the body's ability to produce natural, good quality tears, while quelling the underlying inflammation of dry eye disease.

For the first time we can prescribe a treatment with the knowledge that our patients are likely to return on their follow-up visit happy and satisfied -- and that treatment may actually lighten the economic burden for everyone concerned.

Dr. Donnenfeld practices at the Manhattan Eye, Ear and Throat Hospital. He is also a founding member of Ophthalmic Consultants of Long Island, Rockville Centre, N.Y. He can be contacted at eddoph@aol.com.

Keeping it Simple: What to Tell Patients

Explaining the etiology of dry eye disease to patients can be confusing and time-consuming. In addition, it's important to manage patients' expectations regarding Restasis. I emphasize the following points:

- I tell my patients that Restasis is the first and only FDA-approved product that will increase the body's ability to produce natural, healthy tears by treating the cause of dry eye, not just the symptoms.
- I always explain that Restasis will begin helping to relieve their symptoms within a few weeks or a month.
- Until relief is obtained, artificial tears (e.g., Refresh) can be used intermittently with Restasis. (I tell them to wait at least 15 minutes after using Restasis before using an artificial tear.) I point out that many patients eventually stop using supplemental tears altogether.
- Mild burning is experienced by about 1 out of every 7 Restasis users; I explain to patients that this is normal and will almost always resolve on its own.
- Patients can wear contact lenses when using Restasis, but they need to wait 15 minutes after instilling the drops before putting in their lenses.
- Finally, I make sure patients understand how to use the drops. I tell them to use one vial per day, storing the vial upright in a mug between uses (one drop in the morning, one in the evening -- or visa versa). I tell them to discard the vial after a 24-hour period to avoid contamination and the risk of complications. I also emphasize that, like any medication for a chronic condition, Restasis needs to be used on a long-term basis to ensure that symptoms of dry eye disease don't reappear.

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Case 1:18-md-02819-NG-LB Document 676-14 Filed 04/13/21 Page 31 of 181 PageID #: 70859

палительных изменений слизистой оболочки полости рта и цитологических параметров.

При оценке субпопуляций лимфоцитов в обеих группах по сравнению с контролем выявлены снижение содержания теофиллиночувствительных и повышение уровня теофиллинрезистентных Т-лимфоцитов (табл. 2). Индекс (отношение числа теофиллинрезистентных Т-лимфоцитов к числу теофиллиночувствительных) при БШ был в 4 раза выше, чем в контрольной группе. После лечения ЭНКАД изменения показателей содержания теофиллинрезистентных и теофиллиночувствительных лимфоцитов в обеих группах были сходными: отмечалась тенденция к нормализации содержания теофиллиночувствительных и теофиллинрезистентных лимфоцитов. Эти данные свидетельствуют об иммуномодулирующем действии препарата ЭНКАД при БШ.

Таким образом, данные клинического и цитологического исследования показали, что при БШ с низкой и умеренной степенью местной активности воспалительного процесса в слизистой оболочке полости рта 2-недельный курс терапии препаратом ЭНКАД является достаточно эффективным. При БШ с высокой степенью местной активности воспалительного процесса в слизистой оболочке полости рта рекомендуется более длительный — 4-недельный курс лечения.

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CLINICAL, CYTOLOGICAL AND IMMUNOLOGICAL CHARACTERIZATION OF THE EFFECT OF ENCAD ON THE ORAL MUCOSA IN SJOGREN'S DISEASE

M. M. Pozharitskaya, T. N. Koryeva, O. V. Makarova, M. E. Shabanova

Summary

A study of the clinical efficacy and the immunomodulating and antiinflammatory effect of ENCAD was conducted in 20 patients with Sjogren's disease (SD) of various degrees of activity of oral mucosa involvement and in a group of 23 healthy controls, with a saline solution as placebo. ENCAD was shown to lessen the expression of the clinical symptoms of xerostomia in both groups of a low and a moderate and high degrees of local activity of an inflammatory process. A tendency to the normalization of regeneration and desquamation in the oral mucosa epithelium was observed against a background of the reduction of an inflammatory reaction. ENCAD produced an immuno modulating effect lowering the blood level of theophylline-resistant and raising the level of theophylline-sensitive T-lymphocytes.

УДК 616.4-008.64-031.3-085.275.4-03

А. А. Дрозос (A. A. Drosos), Г. М. Моцопулос (H. M. Moutsopoulos)

ЭФФЕКТИВНОСТЬ И БЕЗОПАСНОСТЬ ПРИМЕНЕНИЯ ЦИКЛОСПОРИНА А ПРИ ЛЕЧЕНИИ ПЕРВИЧНОГО СИНДРОМА ШЕГРЕНА

Отделение ревматологии, Медицинский департамент, Медицинская школа, Университет в Иоаннине, Греция

Первичный синдром Шегрена (ПСШ) — хроническое аутоиммунное заболевание, характеризующееся развитием спектра проявлений от экзокринопатии до системного заболевания и даже лимфоидной неоплазии [14]. Большинство клеток, инфильтрирующих малые слюнные железы при ПСШ, — активированные Т-лимфоциты, имеющие Т-хелперный фенотип [15].

В настоящее время еще не разработаны такие методы лечения, которые бы полностью контролировали лимфопролиферацию. Для лечения ксеростомии используются средства, замещающие недостаток слюнного секрета, применяются бромгексин, муколитический агент [19], пилокарпин-м-холиномиметик [8]. Кортикостероиды не влияют на патологический процесс, а цитотоксические агенты могут повышать риск развития у больных лимфомы [13].

Циклоспорин А (ЦиА) — новый иммуномодулирующий агент, ингибирующий продукцию Т-хелперами интерлейкина-2 [1, 4]. Первоначально ЦиА с хорошими результатами использовали при трансплантации различных органов и костного мозга [7, 20]. Его назначали также при различных аутоиммунных ревматических заболеваниях, однако результаты лечения были противоречивы [2]. Тот факт, что в основе иммунорегуляторных нарушений при ПСШ лежит повышенное накопление Т-хелперов в органах-мишенях и что ЦиА действует именно на Т-хелперы, побудил нас изучить эффективность ЦиА при ПСШ.

Материал и методы. У 20 больных с ПСШ (19 женщин и 1 мужчина), давших согласие на исследование, в течение 6 мес было проведено двойное слепое испытание ЦиА в сравнении с

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плацебо. У всех пациентов выявляли два из трех следующих критериев ПСШ: сухой кератоконъюнктивит, подтвержденный сниженным тестом Ширмер (5 мм/5 мин и менее) и/или положительным результатом при осмотре роговицы со щелевой лампой после нанесения бенгальского розового; ксеростомию, подтвержденную сниженным тестом стимулированного слюноотделения (1 мл/5 мин и менее из железы) и рецидивирующим увеличением околоушных желез при обследовании или в анамнезе. Всем больным производили биопсию малых слюнных желез губы; результаты классифицировали по T. Tarpley и соавт. [21]. Критериями исключения являлись предшествующая терапия иммуносупрессивными, стероидными или нестероидными противовоспалительными препаратами или другими средствами, взаимодействующими с ЦиА [5], злокачественные новообразования в анамнезе, печеночная и почечная недостаточность, гипертензия, хронический алкоголизм. У 1 больного был мембранозный нефрит с нефротическим синдромом (суточное выделение белка 4 г), связанный с ПСШ. Все пациенты прошли полное клиническое и лабораторное обследование перед началом лечения. Побочные эффекты тщательно оценивались с особым акцентом на гипертрихоз, гингивит, тремор, парестезии и другие известные для ЦиА побочные проявления [18]. Клиническая оценка результатов лечения в процессе наблюдения включала тщательный клинический осмотр больных и лабораторные тесты (полная формула крови, печеночные ферменты, креатинин сыворотки, анализ мочи и суточное выделение белка). В течение первых 2 мес все исследования проводили каждые 2 нед, а затем — ежемесячно. Пациенты рандомизированно получали внутрь либо ЦиА (5 мг/кг в день), либо идентичное по виду плацебо. В конце исследования повторяли тест Ширмер, тест стимулированного слюноотделения и биопсию малой слюнной железы, а на 7-м месяце у 8 пациентов произвели биопсию почки с целью исключения нефротоксичности.

По окончании двойного слепого исследования лечение ЦиА в той же дозе продолжали в открытом опыте. 9 больных, получавших ЦиА в течение 6 мес, и 9 пациентов из группы получавших плацебо согласились войти в дополнительное открытое испытание еще на 6 мес. Дальнейший контроль проводили по схеме, описанной выше, а по завершении исследования производили биопсию малой слюнной железы. 10 пациентов (средний возраст 47±8,5 года, длительность заболевания 8,5±5 лет) получали ЦиА, 10 (соответственно 54±11,2 года и 9,2±8 лет) — плацебо.

Результаты и обсуждение. В табл. 1 представлены изменения субъективных симптомов со стороны рта и глаз. У 7 из 10 больных, леченных ЦиА, отмечено значительное субъективное улучшение ксеростомии ($p<0,01$), однако признаки

Таблица 1

Влияние лечения ЦиА на субъективные и объективные проявления ПСШ по сравнению с плацебо в двойном слепом исследовании

Проявления	ЦиА		Плацебо	
	А (n=10)	Б (n=10)	А (n=10)	Б (n=10)
Субъективные				
Ксеростомия	9	2*	8	6
Ксерофтальмия	10	3	9	9
Гипертрофия околоушных желез	5	4	6	5
Объективные (M±m)				
Тест Ширмера, мм/5 мин	4,8±6,2	5,2±10,0	7,3±5,3	5,0±5,1
Околоушная секреция, мл/5 мин	0,5±0,5	1,1±1,2	0,6±0,6	1,0±1,1
Биопсия губы, баллы	1,6±0,7	1,8±1,1	1,9±0,7	2,8±0,8**

Примечание. Здесь и в табл. 2: А — до лечения, Б — после него. Одна звездочка — $p<0,01$, две — $p<0,05$.

ксерофтальмии и выраженность гипертрофии околоушных желез остались без изменений в обеих группах. Предварительный и окончательный тесты Ширмер-1 и стимулированного слюноотделения не выявили значительных различий.

Гистологические изменения со стороны малых слюнных желез губы у больных, получавших ЦиА, не изменялись, а у больных, получавших плацебо, отмечено прогрессирование. Основные лабораторные показатели не изменялись. У 6 из 10 пациентов, принимавших ЦиА, наблюдался гирсутизм, у 1 — умеренная гипертензия, у 1 — гипертрофия десен и у 1 — тошнота и рвота.

Завершили 6-месячное лечение ЦиА 7 больных, 12-месячное — 8. Несмотря на субъективное улучшение со стороны ксеростомии, у 5 из 7 пациентов, леченных в течение 6 мес, и у 7 из 8 пациентов, леченных в течение 12 мес, объективные показатели экзокринной функции не претерпели изменений (табл. 2). Гистологические изменения со стороны малых слюнных желез губы через 6 мес также фактически остались без изменений, но после 12-месячной терапии ЦиА об-

Таблица 2

Влияние лечения ЦиА на субъективные и объективные проявления ПСШ в открытом исследовании

Проявления	ЦиА, 6 мес		ЦиА, 12 мес	
	А (n=9)	Б (n=7)	А (n=9)	Б (n=8)
Субъективные				
Ксеростомия	6	1	8	1*
Ксерофтальмия	9	4	9	4
Увеличение околоушных желез	5	2	5	4
Объективные (M±m)				
Тест Ширмера, мм/5 мин	5,1±6,5	6,4±7,2	4,9±6,8	5,3±10,0
Околоушная секреция, мл/5 мин	1,2±1,1	1,1±0,9	0,5±0,5	0,8±1,2
Биопсия губы, баллы	2,9±0,8	2,3±0,7	1,8±0,7	3,0±0,9**

Примечание. Одна звездочка — $p<0,01$, две — $p<0,01$.

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Таблица 3

Побочные эффекты ЦиА в открытом исследовании

Побочные эффекты	Лечение ЦиА		Срок появления побочного эффекта, мес
	6 мес	12 мес	
Гипертрихоз	6	8	2—10
Гипертензия	1	3	1—9
Инфекции	0	5**	1—8
Тремор, парестезии	2*	0	1
Тошнота, рвота	2*	0	1
Диспепсия, анорексия	2*	1	1
Гипертрофия десен	0	1	5
Кожная сыпь	1	0	4

* У 2 больных прекращено лечение.
** У 1 больного прекращено лечение.

наружена существенная положительная динамика.

Наиболее частым побочным эффектом был гипертрихоз (у 14 человек), появлявшийся на 2—10-м месяце лечения и исчезавший через 2 мес после отмены ЦиА (табл. 3). У 4 пациентов через 1—9 мес от начала приема ЦиА выявлена незначительная гипертензия (систолическое артериальное давление 150 мм рт. ст., диастолическое — 90 мм рт. ст.). У 5 пациентов через 1—8 мес отмечены инфекционные осложнения приема ЦиА. 3 больных прекратили прием ЦиА: 2 человека из-за тошноты, тремора и парестезии в 1-й месяц лечения и 1 больной из-за развития herpes simplex на 8-м месяце лечения. Все побочные эффекты исчезли после отмены ЦиА. Проводимые в динамике лабораторные тесты не менялись ни во время лечения, ни после него, а у больного с нефротическим синдромом наблюдалось снижение протеинурии с 4 до 1,5 г/сут.

Из 8 больных, которым производили биопсию почки, 5 получали ЦиА в течение 7 мес и 3 — в течение 1 мес. В ткани почек не было выявлено каких-либо специфических повреждений или известных для ЦиА признаков нефротоксичности. Изменения заключались в увеличении мезангиального матрикса за счет депозитов IgM и C3 (у 4), развития гиалиновых артериосклеротических изменений с обнаружением в них IgM или C3-депозитов (у 3), небольшой атрофии клубочков и интерстициальном фиброзе (у 3), а также в обнаружении тубулярных вакуолей и/или телец (включений, не идентифицированных как лизосомы при электронной микроскопии (у 6). У больного с нефротическим синдромом, развившимся еще до начала терапии ЦиА, биопсия вновь подтвердила наличие мембранозной нефропатии.

Обсуждая полученные результаты, необходимо отметить, что эффект лечения ЦиА при аутоиммунных заболеваниях неоднозначен [2]. У большинства больных с увеитом, рефрактерным к кортикостероидам и/или цитотоксическим препаратам, наблюдался хороший результат при лечении ЦиА [16, 17]. В то же время результаты лечения офтальмопатии при болезни Грейвса были разными [11, 22]. У 5 больных системной красной волчанкой, лечившихся в течение 7 нед, не отмечено улучшения [12]. Эффективность ЦиА при ревматоидном артрите колеблется от оценки «целесообразно» [6, 10] до «бесполезно» [9].

Как показал двойной слепой метод лечения, малые дозы ЦиА улучшали субъективные симптомы ксеростомии, в то время как гистологические изменения малых слюнных желез губы оставались стабильными. Объективные показатели слезной и околоушной секреции не улучшились. Это расхождение можно объяснить двумя причинами. Во-первых, даже у здоровых людей наблюдается большая вариабельность слюноотделения, зависящая от фазы менструального цикла, времени дня, приема пищи, эмоций и других факторов [3]. Влияние этих факторов трудно поддается контролю, и они могут иметь место у обследованных больных. В то же время незначительность динамики слезной и околоушной секреции может быть связана с малой продолжительностью лечения. Чтобы исключить последнее, мы продлили исследование до 12 мес, но уже в открытом контролируемом режиме.

По мере продолжения лечения отмечено дальнейшее улучшение субъективных признаков ксеростомии, но ни один объективный показатель экзокринной секреции не изменился. Еще более важен тот факт, что 12-месячная терапия ЦиА не подавляет прогрессирование гистологических изменений в малых слюнных железах, несмотря на явную стабилизацию уже через 6 мес лечения.

В наших недавних исследованиях было показано, что поражение малых слюнных желез у больных с ПСШ, леченных ЦиА, выражается в уменьшении общего числа Т-лимфоцитов и Т-хелперов, в то время как количество Т-супрессоров и В-клеток остается стабильным. Эти данные позволяют предположить, что цитотоксические Т-клетки и/или В-клетки играют роль в прогрессировании поражения малых слюнных желез.

Побочные эффекты ЦиА были обычными, и 3 из наших больных вынуждены были прервать лечение. Наиболее часто встречался гипертрихоз, исчезающий через 2 мес после прекращения терапии. Небольшая гипертензия у 4 пациентов полностью контролировалась минимальной гипотензивной терапией (гидрохлортиазид по 25 мг/сут) и также была обратимой по завершении исследования. Не отмечено изменений в уровне и клиренсе сывороточного креатинина, возможно, в связи с низкой дозировкой ЦиА, поскольку описанное повышение уровня креатинина сыворотки у больных ревматоидным артри-

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том, [6], вероятно, обусловлено приемом высоких доз ЦиА и одновременным назначением нестероидных противовоспалительных препаратов. Инфекционная патология у 4 пациентов (у 2 с синуситом и 2 с трахеобронхитом) контролировалась соответствующей химиотерапией, а у 1 больного с herpes simplex лечение было прекращено.

При биопсии почки у 8 больных выявлены неспецифические изменения, не позволяющие сделать определенные выводы об острой или хронической нефротоксичности ЦиА.

В заключение следует отметить, что ЦиА, назначаемый в малых дозах, дал лишь незначительный эффект, проявляющийся в уменьшении субъективных признаков ксеростомии, тогда как объективные параметры экзокринной функции остались неизменными, а гистологические изменения малых слюнных желез губы прогрессировали. Несмотря на незначительные изменения в биоптатах почек у наблюдавшихся больных, при лечении препаратом необходимо иметь в виду возможную нефротоксичность. Таким образом, ЦиА неэффективен при лечении ПСШ. Тем не менее дальнейшие исследования на более многочисленных группах больных и с использованием других дозировок ЦиА представляются целесообразными.

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EFFICACY AND SAFETY OF CYCLOSPORINE-A THERAPY FOR PRIMARY SJOGREN'S SYNDROME

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Summary

We used oral cyclosporine-A (CyA) (5 mg/kg/day) initially in a double blind study for 6 mos. 10 patients received CyA and 10 placebo. At the end of this study it was observed that CyA improved subjective xerostomia, while subjective xerophthalmia, parotid gland enlargement, Schirmer-I-test and parotid flow rate did not show any significant differences in the two study groups. 9 of 10 patients who had received CyA for 6 mos and 9 of 10 in the placebo group continued in an open trial (CyA at the same dose) for an additional 6 mos. At the end of the study the only efficacy of CyA observed was improvement of subjective xerostomia. The side-effects observed were hypertrichosis (14 persons), mild hypertension (4), infections (5) and 3 dropped out because of nausea, tremor, paresthesias and infections. In conclusion, small doses of CyA for 12 mos are rather ineffective for Sjogren's syndrome.

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СРАВНИТЕЛЬНАЯ ОЦЕНКА РАЗЛИЧНЫХ МЕТОДОВ ЛЕЧЕНИЯ БОЛЬНЫХ С БОЛЕЗНЬЮ И СИНДРОМОМ ШЕГРЕНА НА ЭТАПАХ СТАЦИОНАР — ПОЛИКЛИНИКА

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Лечение больных с болезнью и синдромом Шегрена (БШ и СШ) трудная, до настоящего времени нерешенная задача. Большинство исследователей [1, 2, 4, 7, 8] считают базисной терапией при высокой активности и наличии висцеральной патологии лечение глюкокортикосте-

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Cyclosporin A Therapy in Patients with Primary Sjögren's Syndrome: Results at One Year

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We present our experience with small doses of cyclosporine A (CyA), 5 mg/kg/day, in patients with primary Sjögren's syndrome treated for up to 12 months. Subjective improvement in xerostomia occurred at 6 months of treatment, without objective improvement in any sicca parameters. At 12 months, xerostomia improved slightly, but minor salivary gland histology worsened. We conclude that small doses of CyA in patients with primary Sjögren's syndrome for up to 12 months are rather ineffective.

Primary Sjögren's syndrome (primary SS), a chronic autoimmune disease of exocrine glands, is suboptimally treated with secretion replacement (tear and saliva substitutes). We recently reported a double blind study of cyclosporin (CyA) therapy versus placebo. Small doses of CyA (5 mg/kg/day) given for a period of 6 months were associated with subjective improvement of xerostomia and seemed to retard the progression of the histopathologic lesion (7). However, no objective improvement of lacrimal or parotid secretion was found. For these reasons we expanded the study to a total of 12 months as an open trial and report here our findings.

PATIENTS AND METHODS

This open trial is a continuation of a double blind study of CyA therapy versus placebo the results of which have been recently reported (7).

Double blind study. Twenty patients with primary Sjögren's syndrome were included in the double blind study after appropriate informed consent. Ten patients received CyA and ten placeo.

Open study. Nine of the patients having received CyA for six months and nine in the placebo group, accepted to continue the study in an open fashion for an additional six months. All 18 patients recieved CyA in a dose of 5 mg/kg/day orally.

Inclusion and exclusion criteria. All patients fulfilled two of the following three criteria for primary SS: keratoconjunctivitis sicca, as defined by decreased Schirmer-I-test (≤5 mm/5 min) and/or positive slit lamp examination after Rose-bengal staining; xerostomia, as defined by decreased stimulated parotid flow rate (≤1 ml/5 min/gland); and history or presence of recurrent parotid gland enlargement. All patients had a positive minor labial salivary gland biopsy (≥2+ according to Tarpley's classification) (9). Patients on therapy with immunosuppressive, steroidal or non steroidal anti-inflammatory drugs (NSAID), or with drugs known to interfere with CyA (4), history of malignancy, liver failure, chronic alcoholism, renal failure or hypertension, were excluded from the study. All patients underwent a baseline complete clinical and laboratory evaluation. Side effects were assessed carefully with special reference to hypertrichosis, gingivitis, tremor, paresthesias and other known CyA side effects. Follow up clinical assessment included complete physical examination and the following laboratory tests; complete blood count, liver enzymes, serum creatinine, urinalysis and 24 hour urine protein content as indicated, every two weeks for the first two months and monthly thereafter. At the end of the open study, in addition to clinical and laboratory evaluation, Schirmer-I-test, stimulated parotid flow rate and minor salivary gland biopsy were repeated. Student's *t*-test, χ^2 and Wilcoxon tests were applied for statistical analysis where indicated.

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Table I. *Demograp*

Number of patients
Female/male
Age (years)*
Disease duration (year

*: mean±SD

RESULTS

Demographic data o
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Table II. *Effects of*

Subjective	
Xerostomia	
Keratoconjunctivitis si	
Parotid enlargement	
Objective	
Schirmer-I-test (mm/5 min)	
Parotid flow rate (ml/5 min)	
Lip biopsy score (0-4+)	

*: χ^2=7.09, p<0.01.
**: t= 3.1, p<0.01.

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Table I. *Demographic data—open trial*

	Cyclosporine treatment (months) 6	12
Number of patients	9	9
Female/male	9/0	9/0
Age (years)*	58.0±9.5	52.0±10.0
Disease duration (years)*	8.5±9.0	8.0± 7.0

*: mean±SD

RESULTS

Demographic data of our patients are shown in Table I. Mean age, disease duration and sex were similar in the two groups. Seven completed 6 months and eight, 12 months of therapy. Five of seven patients on CyA for 6 months experienced subjective improvement of xerostomia after treatment (Table II). Seven of eight patients on CyA for 12 months also improved subjectively, with statistical significance ($p < 0.01$). However, the results of the Schirmer-I-tests and the parotid flow rates did not show any significant differences after six and twelve months of treatment. Histopathologically the lesion of the labial minor salivary glands seemed to remain unchanged at 6 months, but were significantly more pronounced after 12 months of CyA therapy (Table II).

Side effects of CyA therapy are summarized in Table III. The most frequent was hypertrichosis, occurring in 14 patients. Mild hypertension with systolic pressure ≥150 mmHg and diastolic ressure ≥90 mmHg was observed in 4 patients. Infections were observed in five patients on CyA for 12 months. Three patients dropped out of the study; two because of gastrointestinal disturbance, tremor and paresthesia during the first month of therapy and one because of herpex simplex infection in the eighth month of treatment.

All side effects were readily reversible after CyA discontinuation. The hemoglobin, leukocytes, platelets, creatinine serum level and clearance, liver enzymes (SGOT, SGPT, alkaline phosphatase), potassium and sodium did not exhibit any significant changes

Table II. *Effects of CyA treatment in sicca parameters (open trial)*

	Before CyA (6 months) Number of patients	After	Before CyA (12 months)	After
Subjective				
Xerostomia	6 (8)	1 (7)	8 (9)	1 (8)*
Keratoconjunctivitis sicca	9 (9)	4 (7)	9 (9)	4 (8)
Parotid enlargement	5 (9)	2 (7)	5 (9)	4 (8)
Objective				
Schirmer-I-test (mm/5 min)	5.1±5.6	8.4±7.2	4.9±6.6	5.2±10.0
Parotid flow rate (ml/5 min)	1.2±1.1	1.1±0.9	0.5±0.5	0.8±1.2
Lip biopsy score (0–4+)	2.9±0.8	2.3±0.7	1.8±0.7	3.0±0.9**

*: $\chi^2 = 7.09$, $p < 0.01$.
**: $t = 3.1$, $p < 0.01$.

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Table III. *Side effects of CyA treatment (open trial)*

Side effects	CyA treatment (6 months) Number of patients	(12 months)	Occurence (months)
Hypertrichosis	6	8	2–10
Hypertension	1	3	1–9
Infections	none	5**	1–8
Tremor, paresthesias	2*	none	1
Nausea, vomiting	2*	none	1
Dyspepsia, anorexia	2*	1	1
Gingival hypertension	none	1	5
Skin rash	1	none	4

*: 2 patient drop-out.
**: 1 patient drop-out.

during or at the conclusion of the study. Likewise, there were no significant changes of rheumatoid factor or antinuclear antibody titers, complement levels (C_3, C_4) and circulating cryoglobulins before and after CyA therapy.

DISCUSSION

CyA is a new immunomodulatory agent which acts by inhibiting interleukin-2 production by T-helper cells (1, 3). It has been used in a variety of autoimmune rheumatic diseases with controversial results (2). It is known that the majority of the infiltrating cells of the minor salivary glands in primary SS patients are activated T-lymphocytes bearing the T-helper phenotype (8). This finding as well as the fact that CyA acts on T-helper cells, prompted us to study the efficacy of CyA in the treatment of patients with primary SS in a double blind study. This study showed that CyA given in small doses for six months improved subjective xerostomia and seemed to retard the histopathologic lesion of the labial minor salivary glands (7).

In the present open trial part, despite subjective improvement of xerostomia at 6 and 12 months of treatment none of the objective indices of exocrine secretion changed significantly. In addition, after 12 months of treatment none of the objective indices of exocrine secretion changed significantly. In addition, after 12 months of therapy the histopathologic lesion in the minor salivary glands showed significant progression, while it appeared stable after 6 months of therapy. Recently we also showed that in the minor labial salivary gland lesion of primary SS patients treated with CyA there is a decrease in the number of total T-lymphocytes and T-helper cells, while the T-suppressor and B-cells remain the same (5). These findings suggest that cytotoxic T-cells and/or B-cells may be responsible for deterioration of the labial lesion in the minor salivary glands. Side effects were observed frequently and caused dropout of 3/19 patients. The most frequent side effect was hypertrichosis, which was completely reversible two months after the end of the study. Mild hypertension was noted in four patients; it was completely controlled with minimal antihypertensive therapy (hydrochlorothiazide 25 mg daily) and disappeared at the conclusion of the study. However, no changes in serum creatinine levels or clearance were noted in these patients. These findings could be attributed to the small doses of CyA used. The increase of serum creatinine levels reported in the treatment of rheumatoid arthritis with CyA may well be due to the higher doses used and the concomitant therapy with NSAID (6). Four patients with infections (two sinusitis, two tracheobronchitis) were completely

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controlled with appropriate chemotherapy, while another patient with herpes simplex infection was dropped out.

The present open trial with limited number of patients suggests that CyA treatment in primary SS patients is in the main ineffective. Although our results are not encouraging, studies of larger patient groups and other CyA dosage schedules may be of some value.

ACKNOWLEDGEMENT

We wish to thank Ms E. E. Papanikolaou for expert secretarial assistance.

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m steroids—will be decreased. If so, it may be that all are really doing is reducing the nominal dose. The side ects may well be the same, and the physiologic effect y remain unchanged.

There is precedent for this theory in the published ex-ience of patients with steroid-treated lung disease. It s noted that when patients were treated with tri-ctyloleandomycin (TAO), it was said to have a steroid-uring effect. Sometimes the effect was dramatic, in owing the steroid dose to drop by 50%. In fact, the in-lence of side effects was unchanged, and it was subse-ently determined that this was because metabolism had en altered, we now know, by selective inhibition of cy-chrome P-450 IIIA. Therefore you want to watch for roid side effects in patients who are using lower doses combination with cyclosporine.

Dr. Ellis. So you are suggesting that the term *steroid-sparing* may give an implication that is not justified.

Dr. Watkins. It's possible. Especially if the effects of cyclosporine appear only in conjunction with steroids, and cyclosporine doesn't appear to be effective on its own, it certainly raises the possibility that all you are doing is lowering the effective daily dose of the steroid without changing the physiological consequences of steroid use. However, this has not been tested.

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Topical cyclosporine for oral mucosal disorders

Drore Eisen, MD, DDS,* and Charles N. Ellis, MD *Ann Arbor, Michigan*

Topical cyclosporine may be effective in the treatment of various oral mucosal disorders. In three open trials and in one double-blind study, a topical formulation of this drug produced significant improvement in oral lichen planus. Cyclosporine blood levels were generally low in these studies and no abnormalities of laboratory values resulted during use. Of six patients with oral bullous diseases treated with topical cyclosporine, four showed a decrease in erythema, partial healing of ulcerations, and a reduction in pain. Three patients relapsed shortly after cyclosporine was discontinued. Four of eight patients with persistent aphthous stomatitis remained virtually free of ulcers during 8 weeks of topical cyclosporine therapy. These results indicate that topical cyclosporine is beneficial as a therapy for oral lichen planus and possibly other mucosal diseases. (J AM ACAD DERMATOL 1990;23:1259-64.)

Trials with topical cyclosporine for diseases that spond to systemic administration of the drug have en consistently disappointing.[1-5] However, intrale-nal injection into plaques of psoriasis causes arked improvement.[6,7] These studies suggest that e ineffectiveness of topical cyclosporine may be tributed to insufficient absorption by the skin.

In contrast, the significant absorptive capacity of

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low of Dermatology Associates of Cincinnati, Cincinnati, Ohio.

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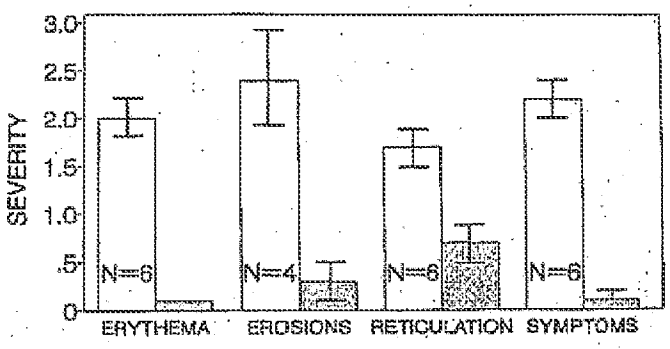

Fig. 1. Clinical improvement of oral lichen planus after treatment with topical cyclosporine. *Open columns:* before therapy. *Shaded columns:* after therapy. *Top of column* indicates mean; *bracket* indicates standard error; *N* = number of patients with condition. Severity scale based on grading each parameter from 0 (absent) to 3 (severe).

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Table I. Results of three open trials with topical cyclosporine (Cs) for oral lichen planus

	No. of patients	Duration of study	Dose of topical Cs	Improvement	Cs blood levels
Eisen et al.[9]	6	8 wk	1500 mg/day swish	Erosion, erythema, symptoms improved by >90% in all patients; reticulation improved 30% to 60% in all patients	20-184 ng/ml measured by HPLC
Frances et al.[11]	4	4 wk	100 mg/day finger rub	Erosion improved by 80% in two patients and 30% to 50% in two patients	30-150 ng/ml measured by RIA
Balato et al.[10]	7	16 wk	100 mg/day × 8 wk then 50 mg/day × 8 wk; method of application unknown	Erosion completely healed in four patients and improved by 40% to 80% in three patients	50-120 ng/ml measured by RIA

HPLC, High-pressure liquid chromatography; *RIA*, radioimmunoassay.

oral mucous membranes, partly facilitated by saliva, is well documented.[8] In view of this, we examined the effects of topical cyclosporine on recalcitrant oral mucosal disorders. This article summarizes our findings and those of others. In all our studies we used cyclosporine in the standard formulation for systemic administration (Sandimmune, 100 mg/ml). By having the patient swish and spit the medication, we achieved a topical mode of drug delivery.

ORAL LICHEN PLANUS

Traditional therapy for oral lichen planus is inadequate. The application of corticosteroids to oral mucous membranes, although inconvenient, remains the most effective form of therapy. Intralesional injections of steroids and topical antimycotics and retinoids are also of benefit. In recalcitrant cases, systemic corticosteroids or retinoids are usually prescribed. However, the long-term use of these agents is limited by their inherent toxicity.

Recently, we[9] and others[10, 11] reported beneficial results with topical cyclosporine in treating oral lichen planus (Table I). Frances et al.,[11] who first published data on this subject, documented improvement of 30% to 80% in four patients with erosive lichen planus of the oral cavity. In this month-long study, patients used their fingers to apply 100 mg of cyclosporine daily to their lesions. Blood cyclosporine levels, measured by radioimmunoassay 2 hours after topical application, ranged from 30 to 150 ng/ml. The improvement in erosive oral lichen planus was corroborated by Balato et al.[10]

We studied six patients with severe oral lichen planus and evaluated not only erosions, as in the aforementioned studies, but also reticulation, erythema, and symptoms.[9] Patients swished 5 ml of cyclosporine (Sandimmune solution, 100 mg/ml for peroral systemic use) and expectorated after 5 minutes. The treatment was repeated three times daily for 8 weeks. All patients improved in all categories evaluated (Fig. 1). Of four patients with erosions, lesions cleared completely in two patients and marked improvement was shown in two (Fig. 2). By 4 weeks, erythema was noted to be significantly reduced in most patients, and continued improvement occurred during the 8 weeks of treatment. Reticulated lesions on the lips and buccal mucosa generally responded better than reticulated lesions on the gingivae and tongue. Significant discomfort, which had been present in all patients before therapy, was greatly diminished after treatment; four of six patients experienced complete relief of pain.

There were no systemic side effects and no laboratory value abnormalities. In most cases, blood cyclosporine levels as measured by high-performance liquid chromatography were low (50 to 90 ng/ml) or undetectable; in two patients, maximal levels of 176 and 184 ng/ml were recorded.

The mechanism by which cyclosporine improves

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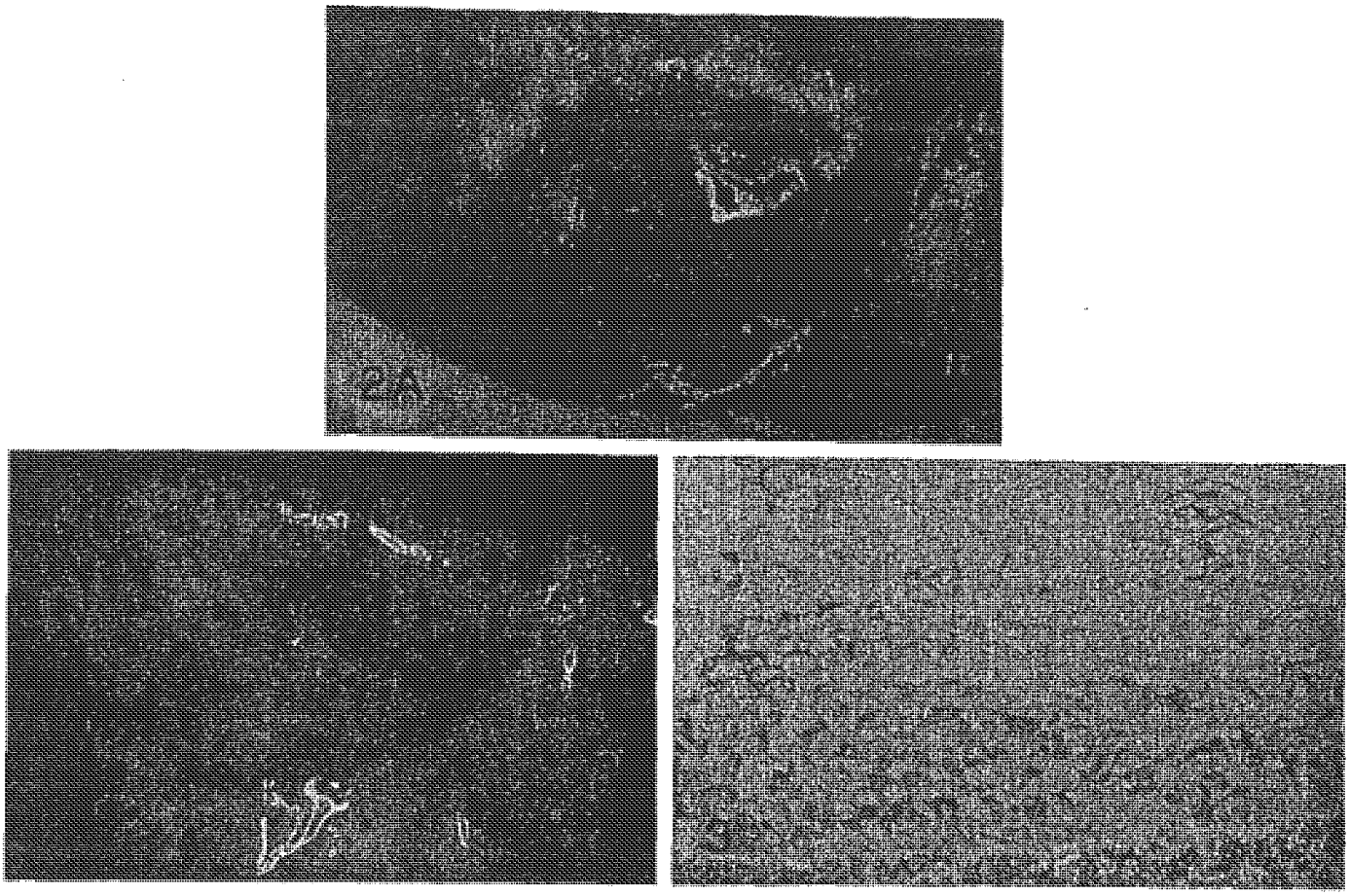

Fig. 2. A, Pretherapy ulceration on ventral surface of the tongue. B, Complete clearing after 8 weeks of cyclosporine swish-and-spit therapy.

Fig. 3. Pretreatment biopsy specimen shows abnormal expression on keratinocytes of ICAM-1.

al lichen planus is unknown. However, the drug ectively inhibits production of interferon-γ, a mphokine thought to be critical in the pathogenis of lichen planus.[12] Produced by activated T mphocytes, interferon-γ induces the expression of tercellular adhesion molecule-1 (ICAM-1) and LA-DR on the surface of keratinocytes. These rface molecules and their interaction with interron-γ trigger an influx of T lymphocytes into the al mucosa, where a cytotoxic inflammatory reacn is established.[13]

We examined the effects of cyclosporine on interron-γ-dependent keratinocyte surface molecules d the associated lymphocytic infiltrates in oral lien planus. In four patients, biopsies of lesional ccal mucosa were performed before and at the end cyclosporine therapy. Epidermal and dermal mphocyte infiltrates were moderately reduced af- therapy, as was HLA-DR expression by keratinocytes and lymphocytes. The expression of keratinocyte ICAM-1, which was present abnormally in pretreatment biopsy specimens (Fig. 3), was virtually undetectable after therapy. The clinical improvement we observed may directly relate to cyclosporine's ability to inhibit these events.

We have recently confirmed the efficacy of topical cyclosporine for oral lichen planus in a double-blind study.[14] Sixteen patients with symptomatic oral lichen planus received topical cyclosporine (100 mg/ml) or its vehicle for 8 weeks. The eight cyclosporine recipients exhibited marked improvement in erythema ($p = 0.003$), erosion ($p = 0.02$), reticulation ($p = 0.007$), and symptoms ($p = 0.002$).

Vehicle-treated patients showed no change or minimal improvement; after switching to cyclosporine, these patients showed improvement comparable to the magnitude attained by patients who initially received cyclosporine. In the majority of

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patients, blood cyclosporine levels were low or undetectable.

ORAL BULLOUS DISORDERS

Involvement of oral mucous membranes with cicatricial and bullous pemphigoid and pemphigus vulgaris is often more severe than that seen in lichen planus. Treatment of the bullous diseases is difficult because those afflicted are usually elderly and the systemic agents required to control disease activity are potentially toxic. Moreover, as is true in oral lichen planus, spontaneous remissions are rare.

In six patients who suffered from severe oral blistering diseases, we initiated treatment with a cyclosporine swish (1,500 mg/day).[15] Two patients with cicatricial pemphigoid and one with bullous pemphigoid used cyclosporine without concurrent systemic or topical therapy. Erythema was moderately reduced but still was present at the end of 8 weeks of treatment. However, both erosions and pain were significantly improved. Two of these three patients had relapses after cyclosporine was discontinued.

Of three patients whose pemphigus vulgaris was treated with topical cyclosporine, one was withdrawn from the study after only 3 weeks because cyclosporine failed to control an exacerbation of his disease. The second patient, whose prednisone dose was reduced from 60 to 40 mg before the study, showed partial healing of extensive erosions and a significant reduction in pain. This patient required a second immunosuppressive agent and an increased prednisone dose shortly after discontinuing cyclosporine therapy. A third patient, who had a maintenance dose of 20 mg of prednisone and 150 mg of azathioprine, showed marked improvement with complete healing of erosions. One month after cyclosporine was stopped, new ulcerations developed.

APHTHOUS STOMATITIS

Eight patients with severe aphthous ulcerations were enrolled in an 8-week trial of topical cyclosporine (500 mg swish, three times daily). To determine the severity of their disease, we required patients to be medication free for 1 month before the study. Of four patients who had averaged more than 12 new aphthous ulcers during the 1-month pretreatment observation period, two reported few (between one and three) and two reported no new ulcers during 8 weeks of therapy. Ulcers that developed during therapy were typically smaller and less painful than those observed before therapy. Two patients had relapses shortly after discontinuing cyclosporine and two others remained free of aphthous ulcerations at a 7-month follow-up.

Of the remaining four patients, two exhibited a lack of response to cyclosporine and two were withdrawn from the study because new, painful ulcerations developed after 3 to 4 weeks of treatment.

Although the pathogenesis of aphthous stomatitis is unknown, evidence in some cases suggests an immunologic basis.[16] The diverse origins of this disorder may be responsible for patients' inconsistent responses to treatment with topical cyclosporine.

DISCUSSION

It has been postulated that local immunologic surveillance involving Langerhans cells, T lymphocytes, and keratinocytes takes place in the oral cavity.[17] Various defects in this mechanism may be crucial in the development of oral mucosal disorders.[18] Topical and systemic retinoids and steroids alter mucosal immunocompetent cells[19] and may correct immunologic defects.

In the absence of substantive systemic absorption, the demonstrated efficacy of intralesional cyclosporine in treating psoriasis indicates that this compound may act by a local mechanism.[7] Similarly, topical cyclosporine may exert local immunomodulatory effects in the oral cavity, as evidenced by the reduction in interferon-γ-induced keratinocyte surface molecules in oral lichen planus. Despite their low or even undetectable cyclosporine blood levels, the clinical improvement in four of our six patients with oral lichen planus supports our hypothesis that cyclosporine acts locally. Even so, a concomitant systemic effect cannot be excluded.

The purpose of these studies was not to develop an optimal protocol for the treatment of mucous membrane disorders. Instead, our aim was to observe the effectiveness of topical cyclosporine. Furthermore, positive clinical responses to cyclosporine may be beneficial in elucidating the pathophysiology of these diseases. Double-blind studies are indicated to assess objectively the efficacy of topical cyclosporine in oral bullous disorders and recurrent aphthous stomatitis.

Although the high cost of cyclosporine at the doses that we used may limit its use as a monotherapeutic agent, these preliminary results warrant

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ther investigation. At lower doses and lower cost, in combination with other agents, cyclosporine ish therapy may offer an alternative in the treatnt of recalcitrant mucous membrane disorders. addition, our studies suggest that if adequate pertaneous absorption could be achieved, cyclospoe's use as a topical therapeutic agent for cutanes as well as oral disorders may become significant.

Cyclosporine was a gift from Sandoz Research Instite, E. Hanover, New Jersey.

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DISCUSSION

Dr. Fry. I can see the logic of using cyclosporine in oral lichen planus. Why do you think cyclosporine works in pemphigoid and pemphigus? What is the mechanism of action of this drug in these diseases?

Dr. Eisen. It is generally agreed that pemphigus vulgaris and bullous pemphigoid are autoimmune disorders. Cyclosporine exerts its immunomodulatory effects on the preliminary phase of the immune response by the inhibition of interleukin 2 release from helper T cells. One would speculate that its use in autoimmune disorders would be of little benefit because immune alterations are already present when patients require therapy. However, cyclosporine could prevent the emergence of new lesions. Furthermore, if these bullous diseases were mediated by a population of short-lived lymphocytes, then cyclosporine's effect on newly appearing lymphocyte clones would become apparent. Therefore it is not surprising to find a wide variety of autoimmune diseases that have been reported to be responsive to cyclosporine.

Dr. Cooper. In pemphigus vulgaris, I think it is more difficult to speculate because one would postulate interference in an early T-B lymphocyte cooperative mechanism in noncutaneous lymphoid tissue. In pemphigoid, however, a direct activity on leukocyte activation in the skin may occur because activated T cells and macrophages are present in the lesions sometimes along the dermoepidermal junction.

It could be postulated that with high concentrations of cyclosporine, non-T-cell effects may be achieved as well. If enough cyclosporine is given in vitro, some macrophage effector mechanisms may be inhibited, which may be important in the pathogenesis of pemphigoid.

Dr. Ellis. Would you comment on the duration of the remission?

Dr. Eisen. In our open trial, five of six patients treated with topical cyclosporine for oral lichen planus were available for follow-up examinations. All five sustained a relatively prolonged remission. Six months after cyclosporine was discontinued, three patients had new lesions of approximately the same severity as observed before therapy. Two patients remain in remission 8 months after therapy.

Unlike cutaneous lichen planus, spontaneous remission in oral lichen planus is rare. Thorn et al.[1] studied several hundred patients with oral lichen planus and found that only 10% of treated patients underwent remission. A pro-

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longed remission in patients with cutaneous lichen planus treated with systemic cyclosporine has recently been reported.[2] This is in contrast to most other disorders treated with cyclosporine in which relapses are common soon after therapy is discontinued.

Dr. Ellis. Some injected psoriasis lesions showed long-term effects. I wonder whether, when a high local concentration is delivered, cyclosporine is actually working through a different mechanism. Injected intralesionally, we obviously are obtaining high tissue concentrations of cyclosporine.

Dr. Eisen. Some people speculate that oral lichen planus is a result of a local immunologic defect. Whether a high localized concentration of cyclosporine corrects the defect is unknown. However, this may explain the clinical improvement and relatively prolonged remission we observed.

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XP009063045

Topical cyclosporine in a bioadhesive for treatment of oral lichenoid mucosal reactions

An open label clinical trial

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VANCOUVER HOSPITAL AND HEALTH SCIENCES CENTRE, BRITISH COLUMBIA CANCER AGENCY AND UNIVERSITY OF BRITISH COLUMBIA, AND UNIVERSITY OF WASHINGTON, SEATTLE

Oral lichenoid reactions may present as chronic symptomatic mucosal reactions. Lichen planus-like reactions include those associated with drug reactions, graft-versus-host disease after bone marrow transplantation, and idiopathic lichen planus. The mainstay of management is topical steroids; in resistant cases, topical and systemic corticosteroids may be used. We evaluated the use of cyclosporine administered in an adhesive hydroxypropyl cellulose base in patients with oral lichenoid reactions that remained active despite the prior use of high-potency topical steroids and in some cases despite the combined use of topical and systemic immunosuppression. Signs and symptoms of ulcerative oral graft-versus-host-disease improved more than 50% in three of four patients with oral graft-versus-host disease treated with the addition of topical cyclosporine. However, in patients with persistent oral lichen planus less effect was seen with 7 of 14 patients demonstrating a partial reduction in signs and symptoms. The topical use of cyclosporine in a bioadhesive base may represent a useful adjunctive approach in management of oral lichenoid reactions, although dose escalation and placebo-controlled studies are needed. (Oral Surg Oral Med Oral Pathol Oral 1996;82:532-6)

Cyclosporine has been used in management of T-lymphocyte–mediated conditions including inflammatory diseases, immunologically mediated graft rejection, graft-versus-host disease (GVHD), and inflammatory diseases. The mechanism of action may be due to inhibition of T-cell activation that may be involved in inflammatory and immune diseases including lichen planus.[1-3] Activated T-cells produce γ-interferon that increases the expression of ICAM-1 and HLA-DR by keratinocytes, which may result in the adhesion of T-lymphocytes promoting an inflammatory reaction.[3]

Systemic and topical cyclosporine has been studied in dermatology for treatment of a variety of conditions. In psoriasis, which may represent a T-cell–mediated condition, systemic therapy has been shown to be effective; however, topical application shows limited effect.[4, 5] Limited effect of topical cyclosporine has also been seen in management of contact dermatitis and atopic dermatitis.[6] The lack of penetration of topical cyclosporine through intact skin has been implicated in this limited efficacy.

Topical cyclosporine has been shown to be effective in a variety of oral mucosal disorders such as lichen planus, aphthous stomatitis, pemphigoid, and pemphigus.[7-11] In lichen planus, a disease that shares clinical features of lichenoid oral GVHD, oral rinsing with cyclosporine has produced clinical or histopathologic improvement in several studies.[3, 7, 8, 12-15]

Low systemic absorption of topical cyclosporine has been demonstrated in blood studies in some patients; no systemic side effects were reported.[3, 7, 14] Cyclosporine measured in biopsy specimens indicate that drug absorption through the mucosa is needed for a beneficial effect, and low systemic levels suggest a local action of the drug.[7] Pre- and posttherapy biopsies have revealed decreased T-cell infiltrate and reduced expression of ICAM-1 and HLA-DR after topical cyclosporine.[3] In a clinical study of oral GVHD, management was related to local effects as no consistent changes in systemic levels of cyclosporine were demonstrated.[16] The dose of cyclosporine applied topically may be important. One study used a low dose of cyclosporine rinse (100 mg/ml, 2 ml rinse, 15 minutes three times per day) with no improvement[12], whereas a higher dose (100 mg/ml, 5 ml, three times a day) in a controlled double-blind

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study was effective.[14] No systemic side effects or laboratory abnormalities have been identified with topical cyclosporine when used in treatment of oral conditions, and only low or undetectable levels of systemic cyclosporine have been seen.

Topical cyclosporine has been studied in treatment of oral lichen planus[3, 7, 8, 12-18] with improvement reported in several publications.[3, 7, 8, 12-15] Eisen et al.[7] provided topical cyclosporine in a double-blind placebo-controlled design for eight patients. Balato et al.[15] demonstrated improvement in oral erosive lichen planus after topical application of cyclosporine (100 mg/day) that was maintained at a lower dose (50 mg/day) for a second month. The recent study of oral lichen planus by Harpenau et al.[14] compared topical cyclosporine (500 mg, rinsed 5 minutes) with placebo and showed statistically significant improvement in erythema, ulceration, and decreased pain scores with the use of cyclosporine. However, limited effect has been reported in other studies.[13, 17, 18] A study of cyclosporine (Sandimmune 0.05%) in an ointment of 40% hypromellose in soft paraffin to a concentration of 0.025% in patients with recalcitrant lichen planus showed only partial response in less than half of the patients.[13]

Zilactin (Zila Pharmaceuticals, Phoenix, Ariz.) forms a unique topical film form composed of hydroxypropyl cellulose that contains salicylic, boric, and tannic acids. The solubility of hydroxypropyl cellulose is altered by the formation of esters with these three acids and results in the bioadhesive film.[19] Zilactin has been shown to bind to mucosa and to provide pain relief in symptomatic oral ulcers.[20] The application has been reported to form a durable film when applied to mucosa, and when dried after application it did not adhere to adjacent contacting tissues. Moisture control during application was found important in duration of adherence of Zilactin.

The purpose of this open clinical trial was to assess the potential of cyclosporine in Zilactin for management of persisting oral lichenoid reactions.

PATIENTS AND METHODS

Patients were enrolled in an open clinical trial if they had an oral lichenoid mucosal condition that was unsuccessfully managed with therapy including topical corticosteroids and, in some cases, topical and systemic corticosteroids. These patients included 14 biopsy-confirmed cases of idiopathic lichen planus in immunocompetent hosts. The remaining four cases represented lichenoid oral GVHD, diagnosed by medical history, clinical features, and histopathologic diagnosis of GVHD of the oral mucosa in one case, the skin in one case, and of skin and gut in two cases. The patients with lichenoid oral GVHD were recipients of allogeneic bone marrow transplants who were referred for treatment for persistent oral GVHD despite systemic therapy (prednisone and cyclosporine) and topical corticosteroids. All patients had been either prescribed high-potency topical steroids (four patients) or systemic steroids for therapy for a minimum of 2 months.

The oral status was examined and recorded as previously described.[21, 22] Each area of the oral cavity was evaluated for the presence and severity of erythema, lichenoid pattern, and ulceration. The maximum size of the ulcer (length × width) and the total area of ulceration were recorded. The variable of lichenoid pattern, a recognized component of oral GVHD, was included in the assessment as described by Schubert et al.[23, 24] Symptoms were assessed with the aid of visual analogue scales. A global assessment was provided by the patient and provider.

Cyclosporine (100 mg/ml, Sandimmune, Sandoz Pharmaceuticals, East Hanover, N.J.) was added to the alcohol phase of Zilactin to a final concentration of 0.5 mg/dl before completion of the manufacturing process. The patients were instructed to apply the medication four times daily. For bone marrow transplantation patients, there were no changes in systemic medications during the trial. For patients with lichen planus, topical and systemic corticosteroids were not provided during the trial with cyclosporine.

Patients were assessed before starting cyclosporine and then reassessed after 2 to 4 weeks. All patients were followed for a minimum of 1 month.

RESULTS

Patient demographics, oral findings and symptoms, and the effect of topical cyclosporine are shown in Table I. For patients with lichenoid oral GVHD, the mean maximum ulcer size was reduced from 0.69 cm^2 to 0.24 cm^2, the mean total ulcer area decreased from 1.88 cm^2 to 0.25 cm^2, the mean severity of erythema reduced from 2.8 to 1.5 (on a 0 to 3 scale) and the area of mucosa with clinical striations was changed from 38 to 27 cm. The overall clinical improvement as judged by the provider was 75%, and the patient assessment was an improvement of 90%. In patients with lichen planus, the mean maximum ulcer size was minimally reduced from 0.24 cm^2 to 0.19 cm^2, the mean total ulcer area decreased from 0.38 cm^2 to 0.27 cm^2, the mean severity of erythema reduced from 2.5 to 1.9, and the area of mucosa with clinical striations was little changed from 32 to 29 cm.

Ten patients reported a bad taste that persisted for a short duration after the topical application, but none of the patients stopped the use of the test preparation

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Table I. Cyclosporine/Zilactin open label clinical trial on oral lichenoid reactions

Lichen Planus			Pretreatment					Posttreatment						
					Ulcer (mm)					Ulcer (mm)			Overall effect	
Patient number	Age	Sex	Striae (cm)	Erythema	Largest	Total	Discomfort (eating)	Striae (cm)	Erythema	Largest	Total	Discomfort (eating)	Patient (%)	Dentist (%)
1	36	M	26	3	11	22	6	25	3	10	20	5	0	10
2	46	F	10	3	21	62	7	11	2	15	45	3	50	20
3	67	M	30	2	20	60	4	30	2	20	60	4	0	0
4	47	M	7	3	4	12	5	8	3	5	15	5	20	0
5	68	F	30	3	55	22	9	35	3	60	24	8	0	0
6	70	F	25	2	10	10	9	22	2	10	10	8	10	10
7	51	F	45	3	35	70	6	46	3	33	66	6	0	0
8	55	M	20	2	70	70	4	15	1	40	40	2	50	40
9	57	M	42	2	9	9	5	42	1	9	9	5	0	0
10	50	M	25	1	0	0	2	23	0	0	0	1	50	50
11	40	F	6	3	30	25	3	6	2	20	25	3	0	0
12	72	F	11	2	3	3	3	10	1	0	0	3	10	25
13	39	M	85	3	60	114	5	80	2	50	60	3	50	30
14	65	M	100	3	24	48	4	68	1	0	0	0	90	70
Mean totals	54.5	—	32	2.5	24	38	5	29	1.9	19	27	4	24	18
Graft-versus-host disease														
15	29	M	62	3	24	100	8	48	1	16	18	2	90	90
16	22	F	26	3	100	400	4	20	2	20	20	1	80	80
17	33	M	20	2	30	40	7	20	2	12	12	2	90	40
18	45	F	45	3	120	213	8	20	1	48	48	1	80	90
Mean totals	32	—	38	2.8	69	188	7	27	1.5	24	25	1.5	90	75

as a result of the taste complaints. Three patients reported discomfort for a short duration (seconds to minutes), and one of the patients did not continue with applications after 1 month because of the sensitivity and the lack of recognition of benefit.

DISCUSSION

This clinical trial was an open label nonblinded evaluation of topical cyclosporine in Zilactin for management of refractory oral lichenoid reactions. All patients had failed to respond to topical or topical and systemic steroids. During the period of evaluation the only oral treatment was topical cyclosporine.

Improvement that was seen included decreased oral discomfort in 67% of patients and improvement in mucositis in 65% of patients. The maximum size of the mucosal ulceration was decreased in nine patients, the total area of ulcerated mucosa was reduced in nine patients, and erythema improved in 65% of the subjects studied. The presence of oral mucosal striations was minimally changed with topical cyclosporine. Patients with oral GVHD appeared to improve more than those with refractory lichen planus. The improvement in GVHD supports a previous study.[16] The greater improvement in oral lichenoid GVHD than lichen planus may be related to a reduced saliva volume in GVHD, leading to more effective application, as saliva wetting the mucosa may reduce the duration of adherence Zilactin to the mucosa. This finding may also suggest that the etiologic mechanisms of oral lichenoid GVHD may differ from those in lichen planus.

The patients' assessment of their status appeared to be related more to symptoms than to the appearance of the oral mucosa. The overall clinical impression of oral lichenoid GVHD was improved in all four patients in the trial. We have previously reported that oral cyclosporine suspension was effective in the majority of patients with symptomatic oral GVHD that persisted despite systemic immunosuppressive therapy and topical corticosteroids and that oral disease flared in two of three of the patients with high-grade responses after they stopped the topical use of cyclosporine.[16] In the patients with lichen planus, a response of 50% or more was seen in two patients (14%), less than 50% reduction in 43% of patients, and no improvement in 43% of patients. This is consistent with the conflicting reports of topical cyclosporine in treatment of lichen planus.[3, 7, 8, 12-15, 17, 18] Differences in results of topical cyclosporine may be due to differences in the design of the studies, small sample size in most trials, variable frequency of application, variable concentrations of cyclosporine used, and differences in the carrier used for application.

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In our trial, we considered spontaneous remission to be unlikely because all of these patients had refractory oral signs and symptoms despite continuing aggressive topical corticosteroid application, and in four cases topical and systemic prednisone had been prescribed for recalcitrant symptomatic lichen planus.

Prior studies have not identified changes in the systemic levels of cyclosporine with topical use; improvement was felt to be due to direct drug contact with the oral mucosa. This finding supports the hypothesis that cyclosporine applied topically can produce a local effect in oral T-cell–mediated conditions.[1-3, 9-12, 14, 18] Another possible mechanism may be the occlusive or protective effect of the bioadhesive that could prevent continued challenge of the oral mucosa by local agents that may trigger or aggravate the inflammatory reaction.

Initial management of symptomatic oral lichenoid reactions includes removal of stimuli if identified, avoiding local tissue irritants, and application of topical anti-inflammatory agents. The primary modality for management has been that of topical steroids. However, there are cases in which even high potency topical steroids are ineffective in controlling symptoms and mucosal ulceration. In these cases systemic agents are considered, however, even with topical and systemic corticosteroids resistant cases occur. Cyclosporine has been shown to be an effective systemic agent in management of oral GVHD including patients with oral manifestations; it has been identified as a treatment approach for patients with lichenoid oral GVHD with topical application by means of rinsing and holding the oral solution. A means of application to localized sites that persists and maintains the contact of the drug, such as assessed here, may be of advantage. Cyclosporine is an expensive medication, however, use of a low concentration product that results in extended contact time with the area of involvement may provide therapeutic effects.

This open clinical trial is limited by its assessment of few cases and the lack of double-blind design. Clinically and histologically oral GVHD and lichen planus appear similar, however, it is possible that there are differences in the underlying causes of these conditions. The preliminary results of this open label clinical trial suggest that further study is appropriate. In symptomatic cases of lichenoid reactions, symptoms were reduced in 55% of the patients, with a mean decrease of 5/10 on a visual analogue scale. However, when only the patients with lichen planus were considered, the reduction in symptoms was 20%. Lichenoid striations were minimally affected in all patients. In 65% of patients with GVHD, reduction in erythema by approximately by half and reduction in ulceration by half was seen, but limited reduction was seen in these variables in the lichen planus group. As in previous studies there was no indication of side effects from topical application although some patients reported a bad taste and minor sensitivity on initial application. In only one case did the sensitivity with application lead to discontinued topical use by the patient. In cases where taste complaints were present, all patients continued application throughout the duration of the trial. On the basis of these preliminary findings, double-blind clinically controlled trials appear warranted. In addition, an escalating dose trial will be valuable to identify the minimum effective concentration of the cyclosporine.

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CORRECTION

The article "Oral stent as treatment adjunct for oral submucous fibrosis," which appeared in the February 1996 issue (Oral Surg Oral Med Oral Pathol Oral Radiol Endod 1996;82:148-50), was written by Phuc Vinh Le, DDS, Mervyn Gornitsky, BSc, DDS, FDCD(C), and Gerard Domanowski, MD.

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Aachener Dermatologenabend am 19. 3. 1997

Meeting at the Department of Dermatology, RWTH Aachen, March 19th, 1997

Universitäts-Hautklinik der Rheinisch-Westfälischen Technischen Hochschule Aachen
(Direktor: Univ. Prof. Dr. med. H.F. Merk)

Vortrag: Was gibt es Neues zur Biologie des Melanoms; Prof.Dr.med. Eva Bröcker, Würzburg

S. Erdmann, W. Angerstein+, M. Hertl

Pemphigus vulgaris der Mund- und Kehlkopfschleimhaut

Pemphigus vulgaris of the oral mucosa and the larynx

(+Klinik für Phoniatrie und Pädaudiologie des Universitätsklinikums Aachen.
Direktor: Univ. Prof. Dr. med S. Klajman)

Zusammenfassung: Bei einer 42jährigen Patientin mit schmerzhaften Erosionen an der Mundschleimhaut wurde aufgrund des histologischen und immunhistologischen Befundes die Diagnose eines Pemphigus vulgaris gestellt. Zusätzlich war die Larynxschleimhaut befallen. Es wurde eine systemische Immunsuppression mit 150 mg/d Prednisolon und 100 mg/d Azathioprin eingeleitet, die jedoch nur zu einer Teilremission führte. Unter adjuvanter Lokaltherapie mit Cyclosporin A und insgesamt vier Behandlungszyklen mit extracorporaler Photopherese kam es zu einer vollständigen Abheilung der Mundschleimhauterosionen und einer deutlichen Rückbildung der Schleimhautveränderungen am Larynx. Mittlerweile ist die Patientin unter der Erhaltungstherapie mit täglich 10 mg Prednisolon und 100 mg Azathioprin seit 4 Monaten beschwerdefrei.

Summary: We report the case of a 42-year-old woman who developed painful erosions of the oral mucosa and the larynx. The diagnosis of pemphigus vulgaris could be confirmed by histological examination. We started an immunsuppressive therapy with 150 mg/d prednisolone and 100 mg/d azathioprin leading only to a partial remission of the oral lesions. Cyclosporine A was applied topically and additionally an extracorporeal photopheresis was conducted four times. Finally the oral lesions resolved and there were fewer erosions of the larynx. Presently the patient is treated with 10 mg/d prednisolone and 100 mg/d azathioprin and has shown no symptoms for a period of four months.

Anamnese

Im Januar 1996 stellte sich in unserer Poliklinik eine 42jährige Patientin vor, die über Schluckbeschwerden und Schmerzen bei der Nahrungsaufnahme klagte. Fünf Monate zuvor waren erstmalig schmerzhafte Erosionen im Bereich der Mundschleimhaut aufgetreten und bislang symptomatisch mit verschiedenen antiphlogistischen, antiseptischen und analgesierenden Mundspüllösungen behandelt worden, ohne daß es zu einer Abheilung kam.

Hautbefund

An den Schleimhäuten beider Wangen, am Gaumen und am Gaumenbogen zeigten sich multiple, scharf begrenzte, bizarr konfigurierte Erosionen mit gelblich-weißen, nicht abstreifbaren Belägen. Ferner fanden sich am linken seitlichen Zungenrand eine schmerzhafte linsengroße Erosion und sublingual diskrete Vesikel. Die Zunge war grau-weißlich belegt. Die Haut und die übrigen einsehbaren Schleimhäute waren unauffällig.

Befund der Lupenlaryngoskopie

Bei der Erstuntersuchung fanden sich flächenhafte, scharf begrenzte Erosionen, zum Teil mit schmierigen Belägen an der linken aryepiglottischen Falte und an der laryngealen Epiglottis links. Bei der letzten Kon-

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trolluntersuchung nach acht Monaten ließen sich nur noch umschriebene, fibrinbelegte Erytheme an der linken aryepiglottischen Falte nachweisen.

Immunserologische Laboruntersuchungen

Routinelaborparameter unauffällig, BSG 30/62 mm n.W., antinukleäre Antikörper negativ. HLA-Typisierung (serologisch): HLA DR 4,13; DQ 2,7; DRw 52. Western Blot mit PVhis (rekombinantes Desmoglein 3-Protein): Nachweis Desmoglein 3-spezifischer Autoantikörper im Serum der Patientin.

Histologie und direkte Immunfluoreszenzuntersuchung (Biopsie vom Zungengrund)

Ausgeprägte chronisch-aktive, mit Erosionen und Ulzerationen einhergehende Entzündung. Eine Bläschenbildung ist in dem vorliegenden Material nicht mehr zu erkennen. Die direkte Immunfluoreszenzuntersuchung zeigt IgG- und C3-Ablagerungen in den epithelialen Zellzwischenräumen. Ferner finden sich fokal ausgeprägte interzelluläre Ablagerungen von IgM und Fibrinogen.

Therapie und Verlauf

Aufgrund der ausgeprägten Schmerzhaftigkeit der Mund- und Rachenschleimhauterosionen und des deutlich reduzierten Allgemeinzustandes der Patientin behandelten wir initial systemisch mit 150 mg Prednisolon und 100 mg Azathioprin täglich und lokal mit antiseptischen und analgesierenden Mundspülungen. Unter dieser immunsuppressiven Therapie entwickelte die Patientin rechts axillär mehrere Abszesse, die chirurgisch saniert und antibiotisch behandelt werden mußten. Wegen fehlendem Ansprechen auf diese immunsuppressive Behandlung entschlossen wir uns zu einer adjuvanten Lokaltherapie mit Cyclosporin A (als Mundspüllösung dreimal täglich) und zu einer extracorporalen Photopherese unter Beibehaltung der systemischen immunsuppressiven Medikation. Im Verlauf von vier Photopheresezyklen, die in der Klinik für Innere Medizin IV (Direktor: Univ. Prof. Dr. med. R. Osieka) durchgeführt und in dreiwöchigen Abständen wiederholt wurden, heilten die Schleimhauterosionen vollständig ab. Nach Reduktion der Tagesdosis von Prednisolon auf 30 mg und 100 mg Azathioprin konnte die Patientin nach viermonatigem stationären Aufenthalt in die ambulante Weiterbehandlung entlassen werden. In den folgenden 4 Monaten trat kein Rezidiv des Pemphigus vulgaris auf, die Patientin ist unter 10 mg Prednisolon und 100 mg Azathioprin pro Tag beschwerdefrei.

Kommentar

Der Pemphigus vulgaris ist eine durch intraepidermale Blasenbildung gekennzeichnete Autoimmunerkrankung von Haut und Schleimhäuten, bei der Antikörper gegen epidermale Zelloberflächenantigene die Adhäsion zwischen epidermalen Keratinozyten hemmen. Als Autoantigen des Pemphigus vulgaris wurde Desmoglein 3 identifiziert, ein in Desmosomen lokalisiertes Adhäsionsmolekül der Cadherinfamilie, das eine wesentliche Rolle bei der Adhärenz epidermaler Zellen untereinander spielt (1,2,13). Neben den typischen schlaffen Blasen, die in großflächige Erosionen übergehen können, weisen über 50% der klinischen Erstmanifestationen des Pemphigus vulgaris eine Beteiligung der Mundschleimhaut auf (8). Typisch sind schmerzhafte Erosionen der Mundschleimhaut, intakte Blasen finden sich nur selten. Diese Schleimhautveränderungen können einzige Manifestation der Pemphigus vulgaris-Erkrankung bleiben; meist gehen sie jedoch den Hautveränderungen lediglich um Monate oder auch Jahre voraus (5,9,11). Der isolierte Mundschleimhautbefall beim Pemphigus vulgaris bereitet häufig differentialdiagnostische Schwierigkeiten: abzugrenzen sind andere erosive Erkrankungen der Schleimhaut wie habituelle Aphthose, vernarbendes Schleimhautpemphigoid bzw. bullöses Pemphigoid (7), Lichen ruber erosivus, systemischer Lupus erythematodes, Erythema exsudativum multiforme, allergische Kontaktdermatitis oder mechanische Traumata. Die Diagnosesicherung erfolgt gewöhnlich durch den histologischen Nachweis einer intraepidermalen akantholytischen Spaltbildung sowie immunologisch durch den Nachweis gewebegebundener bzw. zirkulierender Autoantikörper (10,11). Auch in unserem Fall erfolgte die Diagnosesicherung durch die histologische Untersuchung und durch den Nachweis von Autoantikörpern gegen Desmoglein 3 im Western Blot. Bemerkenswert ist ferner, daß die Patientin das HLA-Antigen DR4 aufwies, das sich überzufällig häufig bei Patienten mit Pemphigus vulgaris findet (1).

Da der Pemphigus vulgaris im allgemeinen unbehandelt zum Tode führen kann, ist die systemische Immunsuppression die Therapie der Wahl. Meist werden Kombinationstherapien eingesetzt, bei denen Glucocorticosteroide zusammen mit Immunsuppressiva wie Azathioprin, Cyclophosphamid, Methotrexat oder Cyclosporin A gegeben werden. Selbst unter dieser kombinierten immunsuppressiven Therapie gelingt es nicht immer, schwere Verlaufsformen des Pemphigus vulgaris erfolgreich zu behandeln. Ferner ist die hochdosierte immunsuppressive Therapie häufig mit schweren unerwünschten Wirkungen assoziiert, wie z. B. dem Auftreten multipler Abszesse in dem hier vorgestellten Fall.

Dem Einsatz adjuvanter, nebenwirkungsarmer Therapien bei refraktären Verlaufsformen des Pemphigus vulgaris kommt daher eine besondere Bedeutung zu (3,4). Zum einen führten wir eine Lokaltherapie der Mundschleimhaut mit Cyclosporin A durch, die zu keiner nennenswerten systemischen Resorption von Cyclosporin A führte. Die Serumspiegel von Cyclosporin A lagen bei 20 µg/l (therapeutischer Spiegel: 100-300 µg/l). Ferner ermöglichte die extracorporale Photopherese eine systemische Immunsuppression ohne die entsprechenden Nebenwirkungen einer hochdosierten Glucocortocoidtherapie (6,12). Der Wirkungsmechanismus der Photopherese ist unbekannt; man vermutet, daß durch die Ultraviolettbestrahlung peripherer Lymphozyten eine Apoptose autoanti-

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genspezifischer T-Lymphozyten induziert wird (12).

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Schlüsselwörter: Pemphigus vulgaris - Mundschleimhaut - Larynx - Immunsuppressiva - Cyclosporin A - extracorporale Photopherese

Key words: Pemphigus vulgaris - oral mucosa - larynx - immunosuppressive therapy -cyclosporine A - extracorporeal photopheresis

G. Bertram, M. Hertl

Lineare IgA/IgG bullöse Dermatose

Linear IgA/IgG bullous dermatosis

Zusammenfassung: Bei einem 54 Jahre alten Mann entwickelten sich seit einem Jahr Erytheme, pruriginöse Papeln, multiple Erosionen und pralle Blasen am gesamten Integument mit Betonung der Extremitäten. Die histologische und die Immunfluoreszenz-Untersuchung zeigten eine subepidermale Blasenbildung mit bandförmigen IgG-, IgA- und C3-Ablagerungen an der Basalmembran paraläsional und am Blasengrund. Aufgrund weitergehender immunserologischer Untersuchungen wurde die Diagnose eines bullösen Pemphigoids gestellt. Der Nachweis bandförmiger IgA-Ablagerungen ist wegweisend für eine besondere Entität des bullösen Pemphigoids, die sogenannte „lineare IgA/IgG bullöse Dermatose„. Unter immunsuppressiver Therapie mit Glukokortikoiden und Diaminodiphenylsulfon (DADPS) heilten die Hauteffloreszenzen fast vollständig ab.

Summary: A 54-year-old man developed erythema, pruriginous papules, multiple erosions and blisters on the entire integument, since one year. Histologic and immunologic examinations demonstrated subepidermal blistering with linear IgG-, IgA- and C3 deposits at the paralesional basal membrane and at the ground of blister. The diagnosis of bullous pemphigoid could be confirmed by additional immunoserological examinations. The proof of linear IgA deposits indicates a subgroup of bullous pemphigoid, the so-called „linear IgA/IgG bullous dermatosis„. The therapy with glucocorticoids and diaminodiphenylsulfon (DADPS) led to a remission of the skin lesions.

Anamnese

Im August 1995 bemerkte der 54 Jahre alte Patient das Auftreten von Rötungen und leichten Schuppungen am rechten Oberarm. Im Laufe der nächsten Monate entwickelten sich am Stamm zusätzlich stark juckende erosive Hautveränderungen und an beiden Unterarmen pralle Blasen.

Hautbefund

Disseminiert am gesamten Integument zeigten sich Erytheme, pruriginöse, zum Teil exkorilerte Papeln und entzündete, krustig belegte Erosionen (Abb.1). An beiden Unterarmen waren ca. 0,5-1 cm große pralle Blasen auf gerötetet Haut sichtbar. Ferner fanden sich im Glutaal-und Perianalbereich sowie am rechten Schulterblatt flächenhafte randbetonte erythematöse Herde

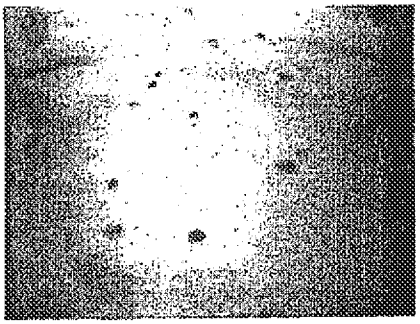

Abb. 1: Pruriginöse Papeln am Stamm bei bullösem Pemphigoid

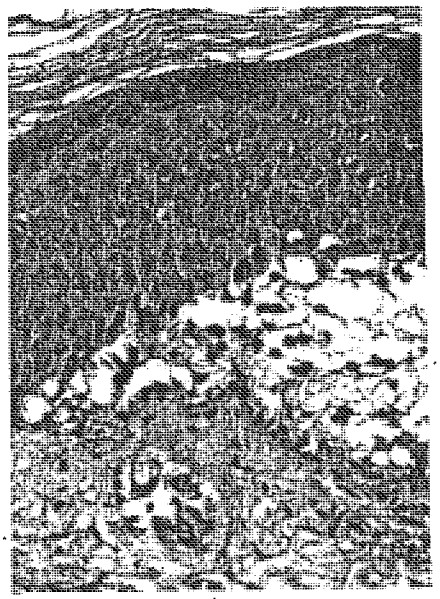

Abb. 2: Blasenwinkel einer subepidermalen Blase bei bullösem Pemphigoid (PAS x 40)

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FDA Concludes Restasis (Cyclosporine) Not Effective for Dry Eye (6/18/1999)

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The FDA Ophthalmic Drugs Subcommittee advisory panel unanimously recommended not to approve Allergan's Restasis for **dry eye** after concluding that clinical trials involving almost 900 patients, did not show efficacy.

Allergan supplied phase 2 and phase 3 study data in its PMA application. Wiley A. Chambers, MD, Deputy Director of Anti-inflammatory, Analgesic and Ophthalmic Drug Products of the FDA chaired the meeting, and by the end of the day the panel had decided that the trials, involving 877 **dry eye** patients, showed no significant difference between cyclosporine treatment and placebo. The studies looked at both **dry eye** symptoms and signs, including corneal staining, and Schirmer testing.

This disappointing treatment study involved more **dry eye** patients than any study preceding it, yet even with all these patients no therapeutic effect for cyclosporine was observed. The company plans on studying more patients to see if a subtle treatment effect was missed.

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CYCLOSPORIN BIOADHESIVE GEL IN THE TOPICAL TREATMENT OF EROSIVE ORAL LICHEN PLANUS

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This study examined the effectiveness of topically applied cyclosporin A (CsA) in the treatment of erosive oral lichen palnus (O.L.P.). The investigation included 20 patients ranging in Age from 31 to 73 years. The patients were divided in two groups: one group of 10 patients were treated with topical cyclosporin in bioadhesive gel for 10 weeks, the other with only bioadhesive gel without cyclosporin for the same period. The study shows remarkable clinical results of efficacy for types of O.L.P. resistant to ttraditional therapy. The blood levels of cyclosporin were very low while, the saliva levels collected in the mouth, were very high.

The relative reduction of relapses, and the absence of side-effects, proves that cyclosporin applied as a topical gel might become a very interesting therapeutic agent in the treatment of erosive oral lichen planus.

Oral lichen planus (O.L.P.) is a chronic disease characterized histologically by a local lymphocytic infiltrate in the chorion. The lesions may assume a variety of morphological changes, and it is possible to recognize 6 types of O.L.P.: papular, reticular, with plaque lesions (quiescent lesions), atrophic, bullous, and erosive (evolutive lesions). The real damage in O.L.P. is due to lymphocytic infiltrate and to the adhesive molecules like ICAM-1 expressed on the tissue by the action of cytokines such as IL-4 and IFN-γ (1).

The tissue lymphocytes presumably appear in response to and react to an

Keywords: Oral Lichen planus, Cyclosporin, Bioadhesive Gel.

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antigen in the epithelium. The antigen expression is probably due to the interaction between endogenous and exogenous agents (viruses, drugs, etc.). The antigen(s) that may be responsible is unkonwn but preliminary studies have revealed a lichen planus specific epidermal antigen (LPSA) in the granular and deep prickle epithelial cells from cutaneous lichen planus (2). Class II antigens also may appear on keratinocytes in lichen planus (3). The therapy of OLP can vary and is related to the severity of the disease (4, 5).

Normally in quiescent types we can only use topical steroids as first choice therapy (betamathasone, fluocinonide or clobetasol) or systemic administration of steroids in low doses (5/6-25/30 mg/die) for deflazacort or prednisolone. In the evolutive types it is necessary to use systemic steroids in high doses (50/60-75/90 mg/die). Sometimes in the evolutive lichen planus when we can not start immunosopressive therapy, we can use topical or systemic retinoids (tretinoine, isotretinoine or acitretine).

The well known and significant capacity of oral mucous membrane absorption induced us to study cyclosporin applied topically and to evaluate its efficacy in the treatment of serious forms of oral lichen planus. Two major features must characterize an O.L.P. formation one is the long term contact with oral mucosa, the other is the ease of its use on patients. Moreover, an ideal bioadhesive drug delivery system should easily stick to the mucous membrane and withstand salivation, tongue movement and swallowing for a period of hours. Such characteristics are not easy to accomplish, that is why in the last decade bioadhesion has received considerable attention from researchers working in the pharmaceutical field who are aware of its potential importance in drug delivery (6-9).

In other studies cyclosporin was used for systemic administration in O.L.P. (6mg/kg) and after 8 weeks of therapy the results were satisfactory (10). Succesively, Eisen et al. in 6 patients (1500 mg/day swish), Frances et al. (100 mg/day finger rub) and in 7 patients Balato et al. (100 mg 7 day in labrafil) used topical cyclosporin in O.L.P. The patients were treated with local applications for 10 weeks and then compared with another group of 10 patients treated with placebo.

MATERIALS AND METHODS

The bioadhesive polymer systems used in this study were prepared from propylene glycol to which sodium carboxymethylcellulose (SCMC) 5% by weight was added. In a typical preparation, 800 mg of cyclosporin in an oil soltion (Sandimmun-Sandoz. Basel.) and 5g of SCMC (Chimisan. Rome.), which were previously ground, and then mechanically mixed with 25 ml of propylene glycol (Carlo Erba. Milan) in a mixer at 300 rpm at 20° C. To this mixture 70 ml of distilled water containing methylhydroxybenzoate (Fadem Naples) 0.15% by weight, as a preservative agent was added. Peppermint water (Fadem. Naples), as flavouring was finally added. Upon complete mixing for 5 min. at 300 rpm at room temperature a viscous gel was obtained.

All chemicals used were from commercial sources, U.S.P. XXII grade.
The criteria for choosing the patients were as follows:
-presence of histologically confirmed oral lichen palnus
-lesions involving more than 50% of the oral cavity
-absence of concomitant therapy to exert additive nephrotoxic effects
-normal liver function and normal creatinemia
-resistance to lacally applied treatments (steroids or tretinoine)

The age of patients ranged from 31 to 73 years. After providing informed consent 10 patetinets were treated with oral cyclosporine for 10 weeks and 10 patients were treated with only the bioadhesive gel without cyclosporin for the same period of 10 weeks.

Treatment protocol

The cyclosporin in bioadhesive gel was applied three times a day (16 mg for each dose, that is 48 mg a day). One a week we controlled blood pressure, complete cells count and differentials, transaminases, bilirubin and alkaline phosphates, blood electrolytes, CsA blood and saliva levels (TDX Abbott), proteinuria. The same protocol was adopted for the control group.

Histological, immunohistochemical and immunofluerescence controls

Punch biopsy specimens were obtained from lesions before and after treatment. Every specimen was divided in three parts. One for histological staining, one was treated with monoclonal antibodies anti HLA-DR (1:10) (Dako, Copenaghen) and the last for immunofluorescence. The same treatment was used for the placebo group.

RESULTS

After 10 weeks of therapy the lesions healed completely in five patients treated with local CsA. In three patients the lesions improved greatly. One patient showed a reduction of about 50% of the mucosa involved. Only one patient showed no improvement since the treatment was discontinued due to local intolerance (this was the oldest patients, 73 years old). No adverse affects were seen except for a transient burning sensation during the first two or three days of local application. The blood levels of CsA were less than 50 ng/ml, while the saliva levels, after three hours from the oral topical application, ranged from 700 to 1500 ng/ml. Biopsy specimens after therapy showed a marked decrease in the interface lymphocytic infiltrate compared with the pre-therapy biopsy specimens and reduction of acanthosis and hyperkeratosis (fig. 1-2).

Immunohystochemical and immunofluorescence results

Before CsA treatment DR II antigen was present in all patients on the surface of keratinocytes, this expression was not found in the control mucosa in normal volunteers at the end of the therapy (10 weeks). The expression of DR II on keratinocytes was reduced moderately (fig. 3-4). Immunofluorescence showed a reduction of fluorescent bodies and the absence of fibrin deposits (fig. 5-8).

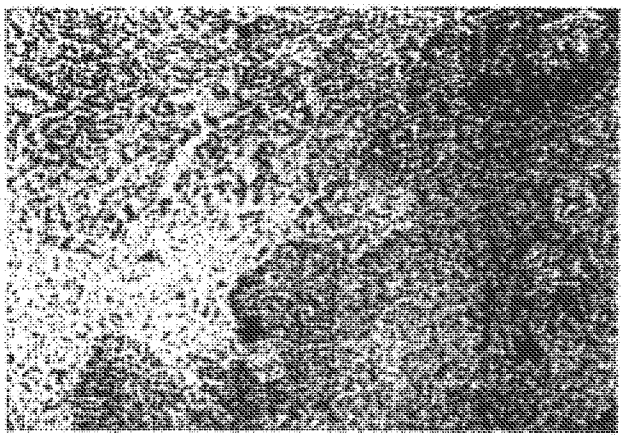

Fig. 1. *Histologic aspects of oral mucosa, thickening of epithelium and lymphocytic infiltrate before treatment with cyclosporin.*

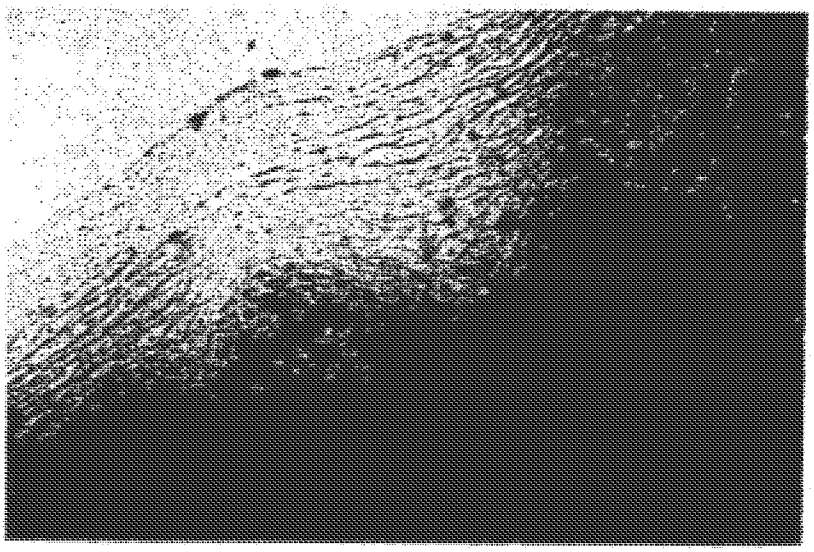

Fig. 2. *Histologic aspects of oral mucosa and reduction of band-like lymphocytic infiltrate after treatment with cyclosporin.*

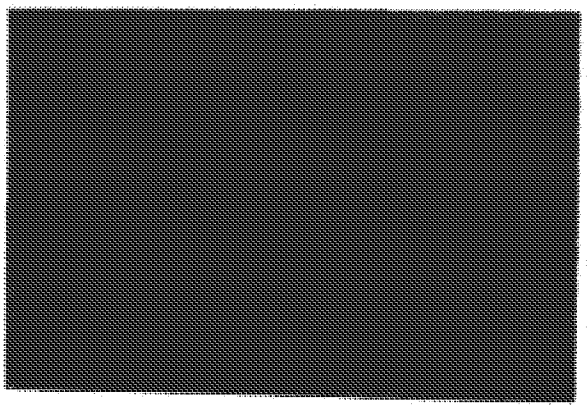

Fig. 3. *Imunohistochemistry: expression of DR antigen in the basal layers before treatment with cyclosporin.*

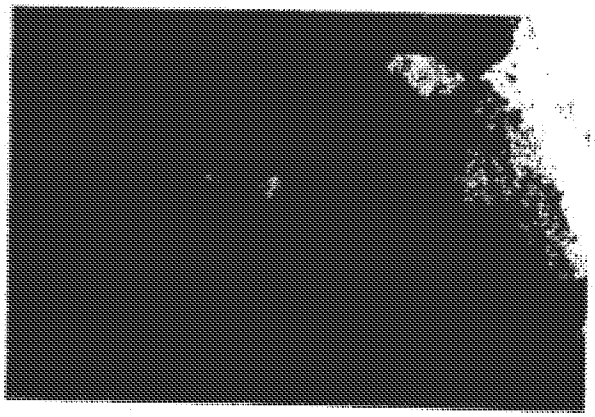

Fig. 4. *Immunohistochemistry: mild reduction of DR antigen after treatment with cyclosporin.*

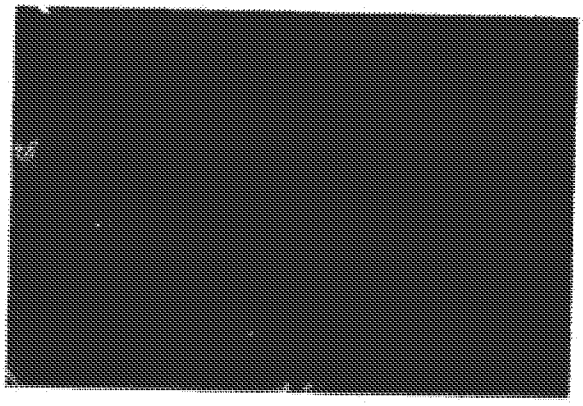

Fig. 5. *Immunofluorescence: presence of fluorescent bodies before treatment with cyclosporin.*

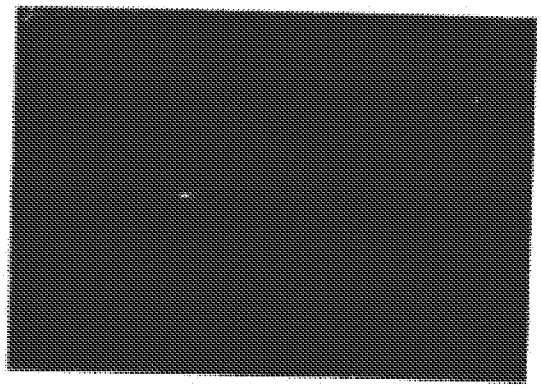

Fig. 6. *Immnuofluorescence: remarkable reduction of fluorescent bodies after CsA treatment.*

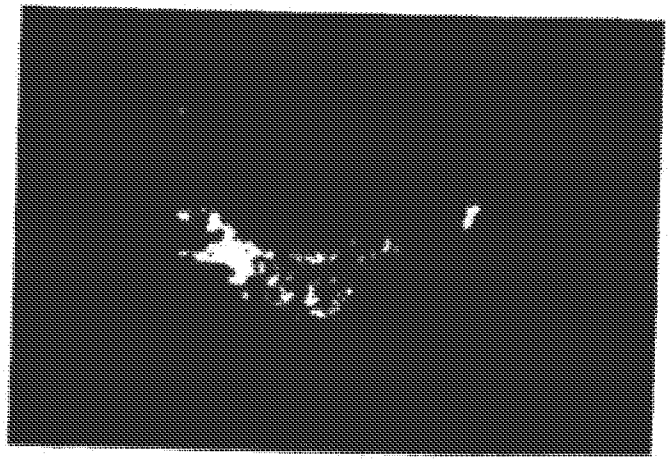

Fig. 7. *Presence of a deposit of fibrin at the dermo-epidermal junction before CsA treatment.*

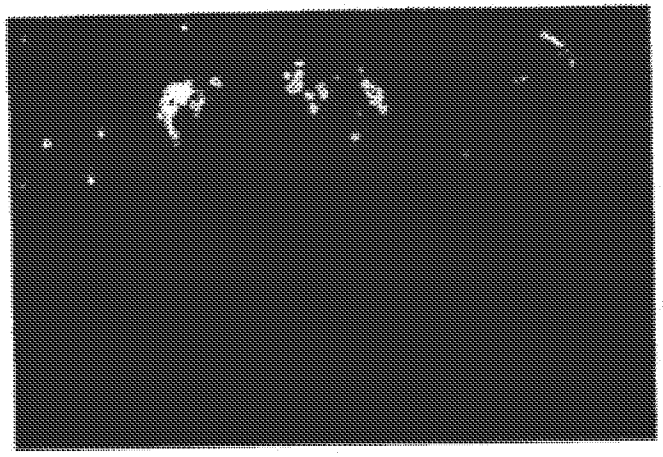

Fig. 8. *Reduction of the deposit of fibrin at the dermo-epidermic junction afterCsA treatment.*

The control group

Two patients asserted that local burning sensation were reduced but no clinical remission was observed. The remaining showed no subjective improvement with the bioadhesive gel without CsA. No hystological, immunohistochemical and immunofluorescence changes were observed before and after treatment.

DISCUSSION

Cyclosporin is a potent immunosuppressant widely used in organ transplantation and various autoimmune disease. The drug has a narrow therapeutic range with various, mostly concentration-dependent side effects, including nephrotoxicity. The immunosuppressive activity of cyclosporin is primarily due to its effect on T cells and B cells. Different sub-populations of T-cells and B cells exhibit different susceptibilities to the drug (15). Cyclosporin blocks the activation of lymphocytes at an early stage, inhibiting lymphokine production by stimulated lymphocytes (16). Therefore it prevents delivery of these essential factors necessary for the proliferation, differentiation and maturation of helper T lymphocytes and other immunocytes (17-18). More specifically, it has been shown to inhibit the transcription of m-RNA for interleukin-2 (IL-2) and interferon-γ (IFN-γ) in vitro (19). The inhibition of IFN-γ which induces HLA-DR expression on keratinocytes, and the production of the intracellular adhesion molecule (ICAM-1) may play a key role in the therapeutic effects of the drug in O.L.P. In this way CsA may exert local immunomodulation in the oral cavity as shown by the reduction of keratinocyte surface molecules in all treated patients.

The blood levels of cyclosporin were very low (0-50ng) if compared to therapeuticl blood dosage of cyclosporine in other immunological diseases. The saliva levels detected after citric acid stimulation (0,5 ml), during the dosage interval, were very high (about 30 times than of blood levels), and reduced 9 hours after drug intake.

This data is in accordance with recent studies (Modèer 1992)(20) in which was demonstrated that when the CsA is administered in a mixture (oil solution) the levels in whole saliva where 130 times higher than the drug administered in capsule form, during the dosage interval.

This observation indicated that CsA administered in bioadhesive gel probably may adhere to the oral mucosa or to the dental plaque for a long time (9 hours).

Thus the vehicle in which the drug is administered should be considered for the local therapeutic effects. This is very interesting for O.L.P. which is a disease that targets exclusively the oral cavity without systemic involvement of the patient's immunity.

Infact, the perpheral blood of patients with O.L.P. are not different from those of healthy controls, differently from the peripheral blood of patients with cutaneous involvement, which are predominantly T helper-inducer (CD4+CD8-) cells.

During the studies we did not observe the side effect of gingival overgrowth due to the drug. The gel has been shown to adhere very well to the mucosa with a resulting prolonged time of contact of the drug and high level of patient acceptability and compliance.

The low cyclosporin dosage utilized (48 mg/day), and the good local absorption without side effects, proves that this bioadhesive gel might become very interesting as a topical agent and as an alternative in the treatment of recalcitrant O.L.P.

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Character of Ocular Surface Mucins and Their Alteration in Dry Eye Disease

ILENE K. GIPSON, PHD, YUICHI HORI, MD, AND PABLO ARGÜESO, PHD

ABSTRACT At the ocular surface, three types of mucins are present. The large *gel-forming* mucin MUC5AC is expressed by conjunctival goblet cells. Some cells of the lacrimal gland acini express the small *soluble* mucin MUC7. The corneal and conjunctival epithelia express the *membrane-associated* mucins MUCs 1, 4, and 16.

With the characterization of the mucin gene repertoire of the ocular surface epithelia, studies of the function of specific mucins, their gene regulation, and their alteration in ocular surface disease have begun. Current information suggests that all the mucins are hydrophilic and play a role in maintenance of water on the surface of the eye. The large secreted mucins represent the "janitorial service" that moves over the surface of the eye to wrap up and remove debris. The membrane-associated mucins form the glycocalyx, which provides a continuous barrier across the surface of the eye that prevents pathogen penetrance and has signaling capabilities that influence epithelial activity. Factors regulating mucin gene expression include retinoic acid, serum, and dexamethasone.

Alteration in both secreted and membrane-associated mucins occur in drying ocular surface diseases. In Sjogren syndrome, MUC5AC expression is reduced, and in non-Sjogren dry eye, glycosylation of MUC16 appears to be altered. The pattern of expression of enzymes that glycosylate mucins is altered in ocular cicatricial pemphigoid. Therapies being evaluated for dry eye, including cyclosporine A, P2Y2 agonists, gefarnate, 15-(S)-HETE, and corticosteroids, may be efficacious due to their effect on mucin gene expression and secretion.

KEYWORDS: dry eye, epithelia, epithelial mucins, mucins, MUC1, MUC4, MUC16, MUC5AC, ocular surface

I. INTRODUCTION

The surface of the eye is covered by a tear film that serves to protect and lubricate the ocular surface, as well as to provide the major refractive surface for the visual system. The epithelial surface of the eye and its specialized glandular infoldings produce the components of the tear film that include water, protective antimicrobials, cytokines, lipids, and mucins (Figure 1A). In addition, the apical surface of the ocular surface epithelia, both corneal and conjunctival, provide a specialized interface between the tear fluid and the epithelium that stabilizes the fluid layer. That interface includes the undulating membrane ridges on the apical cell's apical membrane, termed microplicae (Figure 1B), and emanating from their apices, a layer termed the glycocalyx (Figure 1C). Mucins are present in the glycocalyx layer, as well as in solution within the tear fluid.

Mucins are defined as glycoproteins that are heavily glycosylated, with 50–80% of their mass comprised of carbohydrate. A second characteristic of mucins is the presence in their protein backbones of tandem repeats of amino acids that are rich in serine and threonine, which provide the sites for O-type glycosylation.[1,2]

The heavy glycosylation of mucins is believed to impart a highly negative charge and a hydrophilicity that provides a barrier to pathogen adherence and penetrance into the epithelium.[3] It also provides a lubricating surface that prevents epithelial-epithelial adherence during the blink. Before molecular techniques were applied to the study of mucins, little was known of their biochemical character and their cellular sites of synthesis. These techniques applied to the study of mucins in the last decade have released a flood of new information that has helped greatly in our understanding of the ocular surface.

This review will first summarize the current understanding of mucin protein structure, types, and function. It will then describe the distribution and pattern of expression of mucin genes by the ocular surface epithelia, and, lastly, dis-

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Abbreviations are printed in **boldface** where they first appear with their definitions.

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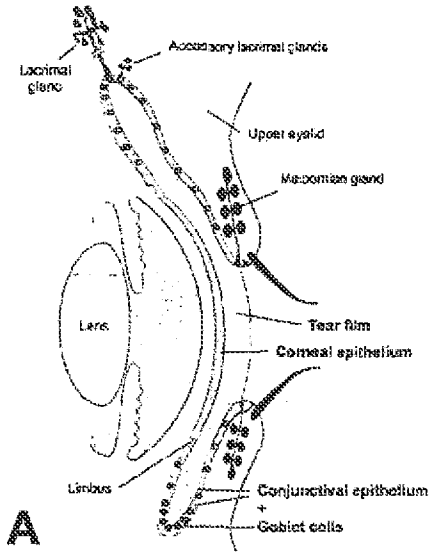
cuss the relationship of the mucins to the tear film. These summaries will introduce the reader to the work that has been done to characterize alterations of mucins in dry eye and also will allow development of new hypotheses for the role of mucins in health and drying ocular surface diseases. A comprehensive review on the topic of mucins of the ocular surface epithelia has recently been published.[1]

II. MUCIN PROTEIN STRUCTURE

Before the molecular characterization of mucins, all mucins were considered to be secreted from specialized cells—usually goblet cells or mucus-secreting cells of glands. Based on recent sequence data, however, it is now known that, in addition to secreted mucins, membrane-associated mucins are present at the apical surfaces of epithelia.[1,2] Table 1 summarizes the mucins identified to date, along with several of their characteristics. Figure 2 demonstrates the major structural features of the secreted and membrane-associated mucins. To date, seven mucins are considered as secreted mucins, ten are membrane-associated, and several mucins remain uncharacterized. Human mucins are designated by "MUC" followed by a number that indicates the chronological order of their discovery.



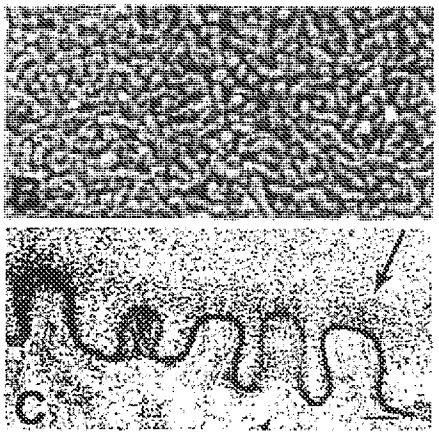


Figure 1. A. Diagram of the ocular surface and adnexia, showing (in various colors) epithelial sites of mucin expression and the tear film. B and C. Scanning electron micrograph B and transmission electron micrograph C of surface ridges or microplicae on the corneal (B) and conjunctival surface (C). Note electron-dense glycocalyx (arrow) at tips of microplicae in C, as well as actin filaments inserting into the same tips from the cytoplasmic face. Bar in B = 10 μm; bar in C = 0.1 μm. (A is reprinted from Gipson and Argüeso[1] with permission of Int Rev Cytol. C is reprinted from Nichols et al[52] with permission of the authors and Invest Ophthalmol Vis Sci.)

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For example, MUC1 was the first of the human mucin genes to be cloned and characterized. Mouse homologues to human mucin genes are designated by "Muc" and rat genes by "rMuc," while the nascent unglycosylated mucin protein is termed "apomucin."

A. Secreted Mucins

Two types of secreted mucins have been identified (Figure 2A)—the large gel-forming mucins and the smaller soluble mucins. (For review, see Gendler and Spicer, 1995,[1] and Moniaux et al, 2001.[2])

1. Gel-forming Mucins

Five large gel-forming mucins have been described; four are encoded on chromosome 11p15.5, and a recently discovered one is present on chromosome 12q12. The gel-forming mucins encoded on chromosome 11p15.5 include MUCs 2, 5AC, 5B and 6, and the one on chromosome 12q12 is MUC19. These mucins are called *gel-forming* because they are responsible for the rheological properties of mucus.[5] They share common structural motifs and are believed to have evolved from a common ancestral gene, similar to the human von Willebrand factor gene—a component in blood that facilitates clotting.[6,7] These gel-forming mucins are expressed by goblet cells of the respiratory, gastrointestinal, endocervical, and ocular surface epithelia, as well as by the mucin cells of the submucosal glands associated with these epithelia. The goblet cells or mucus-producing cells of each of these organ systems have a tissue- and cell-specific pattern of expression of the gel-forming mucins (For review, see Gipson and Argüeso, 2004.[8]) At the ocular surface, the major mucin of this class expressed by the goblet cells of the conjunctiva is MUC5AC (see below).[8]

The gel-forming mucins are probably the largest glycoproteins known. This is borne out by the size of their cDNAs, which range from 15.7 to 17 kb, making the molecular weights of these huge apomucins approximately 600 kDa.[2] The gel formers share several structural motifs (as an example, see diagram of MUC5AC, Figure 2). Each has a large, central, tandem repeat domain flanked by cysteine-rich domains that have homology to the so-called D domains of von Willebrand factor. Three cysteine-rich D domains are present on the amino terminal side of the central tandem repeat, and one on the carboxy terminal side (except for MUC6, which lacks the C-terminal D domain). These domains allow multimerization of individual mucin molecules through disulfide bond formation, which begins in the endoplasmic reticulum, with further multimerization in the Golgi apparatus.[9,10] Multimerization and subsequent glycosylation produces huge macromolecular structures with an estimated molecular weight of up to 40 MDa.[10]

2. Small Soluble Mucins

Mucins in the second category of secreted mucins are the small soluble mucins that include MUC7 (Figure 2A) and MUC9 (also known as oviductin, whose only demonstrated tissue expression is fallopian tube).[11] These mucins lack cysteine-rich D domains and are present predominantly as monomeric species. MUC7 was originally cloned from salivary gland and is one of the smallest mucins known, comprised primarily of tandem repeats of amino acids. It has a histatin-like domain on the N-terminal side of the tandem repeat and lacks cysteine-rich domains. The MUC7 apomucin is 39 kDa,[12] which is secreted by serous cells rather than mucus cells of both the salivary glands[13]

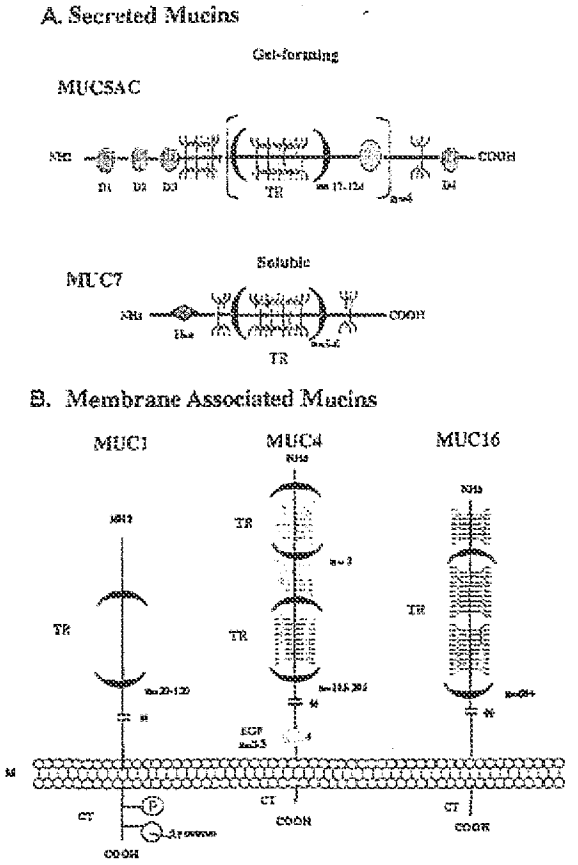

Figure 2. Diagrams demonstrating structural features of the mucins expressed by ocular surface epithelia. All mucins have tandem repeats (TR) of amino acids in their protein backbone that are rich in serine and threonine, which are O-glycosylated (Ψ). Repeats of TRs vary in individuals and alleles. A: Of the secreted-type mucins, MUC5AC, expressed by goblet cells, is a large gel-forming mucin, with D domains that are rich in cysteines and that provide disulfide cross-linking sites for polymerization of these mucins to form the mucous network that gives mucus its rheologic properties. The small soluble mucin MUC7, expressed by acinar cells of the lacrimal gland, has a histatin-like domain (Hsn) in the amino terminal region and does not form disulfide linkages. B: The three membrane-associated mucins, MUCs 1, 4 and 16, are expressed by corneal and conjunctival epithelia. MUC1's cytoplasmic tail has seven tyrosine residues that can be phosphorylated (P) and participate in signal transduction, as well as sites of association with β and γ catenin, which link to the cytoskeleton. MUC4 has EGF-like domains extracellularly. All membrane-associated mucins have cleavage sites (*) associated with shedding of the ectodomain from the surface of the cell.

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and the submucosal glands of the bronchial airways,[14] as well as by acinar cells of the lacrimal gland.[15]

B. Membrane-associated Mucins

As stated above, until molecular techniques were applied to the characterization of mucins, all mucins were assumed to be of the secreted type; however, increasing numbers of mucins are being characterized as having hydrophobic membrane-spanning domains near their carboxy terminus (Figure 2B). These mucins appear to be major constituents of the glycocalyx of cells of all wet-surfaced epithelia—including both simple and stratified—and are estimated to extend 200–500 nm from the cell surface.[16] MUCs 1, 3A, 3B, 4, 12, 13, 15 16, 17, and 20 have been designated as membrane-associated (Table 1). Genes of four of this group (MUCs 3A, 3B, 12, and 17) are clustered on chromosome 7q22 (Table 1).[17–19] Of the membrane-associated mucins, MUCs 1 and 4 have been the most extensively studied (for reviews, see Gendler, 2001,[20] and Carraway et al, 2000[21]). The major part of the extracellular portion of the molecule is occupied by the heavily glycosylated tandem repeat domain that in some instances extends nearly to the amino terminus (Figure 2B). These extracellular domains, also called *ectodomains*, are envisioned as extending from the cell surface to form the glycocalyx.

Table 1. Human Epithelial Mucin Genes

(Those whose expression in ocular surface epithelia has been verified by both mRNA and protein assays, and whose cellular origin is known are shown in ***boldface****.)*

Designation	Type if Sequence Verified	cDNA Clone Source	Chromosomal Mapping	Amino Acids in Tandem Repeat	References
MUC1*	**Membrane-associated**	**Mammary/pancreatic tumor**	**1q21-q23**	**20**	**112,113**
MUC2	Gel-forming/secretory	Intestine	11p15	23	114
MUC3A*Δ	Membrane-associated	Intestine	7q22	17	115
MUC3B*Δ	Membrane-associated	Intestine	7q22	17	18
MUC4Δ	**Membrane-associated**	**Trachea**	**3q29**	**16**	**116**
MUC5AC	**Gel-forming/secretory**	**Trachea**	**11p15**	**8**	**117,118**
MUC5B	Gel-forming/secretory	Trachea	11p15	29	119
MUC6	Gel-forming/secretory	Stomach	11p15	169	120
MUC7	**Soluble monomer/secretory**	**Salivary gland**	**4q13-q21**	**23**	**12**
MUC8		Trachea	12q24.3	13/41	121
MUC9	Secreted, 120 kDa	Fallopian tube	1p13	15	122
MUC11		Intestine	7q22	28	17
MUC12*Δ	Membrane-associated	Intestine	7q22	28	17
MUC13*Δ	Membrane-associated	Intestine, trachea	3q13.3	15	28
MUC15	Membrane-associated	Mammary gland	11p14.3	None	123
MUC16*	**Membrane-associated**	**OVCAR-3 cells**	**19p13.2**	**156**	**39**
MUC17*Δ	Membrane-associated	Intestine	7q22	59	19
MUC19	Secreted	Genomic databases	12q12		124
MUC20	Membrane-associated	Kidney	3q29	19	30

Characteristics of Mucins:
* Have SEA Domains (MUC16 has six SEA Domains—five of them in the tandem repeat region.)
Δ Have EGF-like Domains (MUCs 3A, 3B, & 4 have two EGF-like domains.)
Note: MUCs 14 and 18 do not appear in the HUGO gene nomenclature website: www.gene.ucl.ac.uk/nomenclature, then Quick Gene Search MUC.

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Experimental studies indicate that the extracellular, highly glycosylated tandem repeat domain functions as a "disadhesive," preventing cell-cell and cell-matrix interactions.[22]

The membrane-associated mucins do not have cysteine-rich domains and do not form multimers. They have short cytoplasmic domains (Figure 2B), which in MUC1 is conserved between mammalian species and is reported to be associated with the actin cytoskeleton.[23,24] There is also growing evidence that the cytoplasmic domains interact with cytoplasmic proteins to facilitate signal transduction. Tyrosine residues in the cytoplasmic domains of MUC1 can be phosphorylated, and, thus, through interaction with proteins having SH2 domains, are believed to participate in signal transduction that mediates cell cycle and apoptotic activity.[25,26] Putative tyrosine phosphorylation sites are also present in MUCs 12 and 16.[17,27]

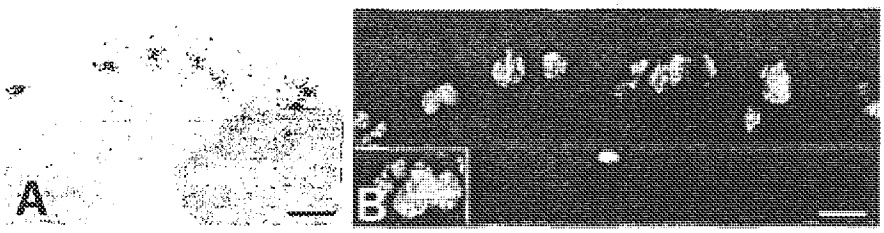

Figure 3. In situ hybridization demonstrating message for MUC5AC in human conjunctival goblet cells (A) and immunohistochemical localization of MUC5AC protein to human conjunctival goblet cells (B). Inset in B shows staining of individual mucin packets. Bars = 50 μm. (Reprinted from Gipson and Argüeso* with permission of Int Rev Cytol.)

In addition to their function as "disadhesives" and signal transducers, the membrane-associated mucins may have other cytokine-like functions. MUCs 3A, 3B, 4, 12, 13, and 17 have two or more epidermal growth factor (EGF)-like domains between the tandem repeat region and the membrane-associated domain.[17-19,21,28] Studies of rMuc4 have provided evidence that the EGF-like domains play a role in the regulation of epithelial growth (for review, see Carraway et al, 2002[29]). The EGF domain of rMuc4 interacts with ErbB2 growth factor, inducing ErbB2 phosphorylation and potentiating neuroregulin-activation of ErbB3 receptor. These receptor interactions suggest that rMuc4 has the potential to be involved in regulation of epithelial cell growth.

Several of the membrane-associated mucins, MUCs 1, 4, 16, and 20, are present in both a membrane-associated and a soluble form.[29-31] The soluble form may be the result of splice variants in which the membrane-associated domain is post-transcriptionally removed[2,20,30] or the ectodomain or α domain of the mucins is shed from the surface of cells.[32] MUC1, MUC4 and MUC16 all appear to be shed from the epithelial surface.[31,33,34]

The mechanism by which the membrane-associated mucins are shed from the apical surface of cells is unclear. Apomucins of MUC1, rMucs 3 and 4 are proteolytically cleaved in the endoplasmic reticulum where they reassociate to form noncovalently bound heterodimers composed of an α (extracellular tandem repeat domain) and a β (includes EGF-like domains, transmembrane domain, and cytoplasmic tail) subunit. The proteolytic cleavage site that produces the subunits is hypothesized to be at a GDPH sequence for rMuc4 (for review, see Carraway et al, 2000[21]), or the so-called sea urchin sperm protein, enterokinase and agrin (**SEA**) module at a region that has the sequence G/SVVV for MUC1[35,36] and rMuc3.[37] Several of the other membrane-associated mucins that have SEA domains include MUCs 3A, 3B, 12, 13, 17,[16-18,28,38] and 16,[39] which has six SEA domains. After reassociation of the α and β subunits, the heterodimer moves through the Golgi for glycosylation and then to the apical membrane of epithelial cells. It is not clear whether the shedding of the ectodomain of the membrane-associated mucins occurs at the association site of the α and β subunits or whether shedding involves a different proteolytic event. The ectodomain of MUCs 1, 4, and 16 have been detected in tear fluid (Spurr-Michaud et al and Hori et al, submitted to ARVO, 2004).

III. PATTERNS OF EXPRESSION OF MUCINS IN OCULAR SURFACE EPITHELIA

The epithelium that covers the entire ocular surface expresses mucins that contribute to maintenance of the tear film. Not only do the goblet cells of the conjunctiva produce mucins, the stratified epithelia of both cornea and conjunctiva, as well lacrimal gland acinar and ductal cells, produce mucins. The pattern of expression and type of mucin produced in the various regions of the ocular surface do, however, vary and may reflect the functions of each epithelial zone.

A. A Major Mucin of the Conjunctival Goblet Cells is the Gel-forming Mucin MUC5AC

Since goblet cells of the conjunctival epithelium have long been considered the major source of the mucus on the ocular surface, it is not surprising that they appear to be the major source of the gel-forming mucins of the tear film. As described above, the gel-forming mucins are the largest of the mucins and give mucus its rheological properties. It seems reasonable that the large gel-formers for the tear film be made in and secreted from the conjunctival goblet cells, since they can secrete directly onto the ocular surface without having to send their large secreted product through a duct system. As demonstrated by in situ hybridization (**ISH**) and immunofluorescence microscopy, the goblet cells of the human conjunctival epithelium express the gel-forming mucin MUC5AC (Figure 3).[8,40] Fluorescence in situ hybridization shows that the

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message for the mucin is located near the goblet cell nucleus, corresponding to the position of the major accumulation of the endoplasmic reticulum of the cell. All the goblet cells in sections of human bulbar conjunctiva appear to bind probes to the MUC5AC gene (Figure 3A), but there has not been a systematic study of all zones of the human conjunctiva, nor have there been studies of the goblet cell crypts located in the forniceal region of the human conjunctiva.[41] Antibodies specific to the D domain of MUC5AC have been developed and their specificity demonstrated.[42] These antibodies localize to the secretory mucin packets of the conjunctival goblet cells (Figure 3B), and they have been used in an ELISA to detect and measure levels of MUC5AC in tears. Pretreatment of tear proteins with neuraminidase to remove terminal sugars enhances the binding of the antibody. Although there is considerable variation from one individual to another in amount of MUC5AC in tears, the levels of the mucin are consistent within an individual over several samplings.[42]

Screening for the expression of three other gel-forming mucins, MUCs 2, 5B, and 6, in conjunctival epithelium has been done by Northern blot and polymerase chain reaction (PCR), but to date there have been no assays for MUC20. Low levels of MUC2 message have been detected by PCR (some 5,600-fold lower than 5AC) in conjunctival epithelial RNA of some individuals,[43,45] but, despite attempts to demonstrate the localization of the MUC2 message by in situ hybridization in both human and rat conjunctiva, no message has been detected. Thus, it is not known if the MUC2 message is translated.[8] PCR has also detected MUC5B message in the conjunctiva, but neither the message nor protein has been demonstrated in the goblet cells.[15]

B. Membrane-associated Mucins of the Stratified Cells of Corneal and Conjunctival Epithelia

The human ocular surface epithelia express at least three membrane-associated mucins—MUCs 1, 4 and 16 (Figure 4).[44-46] Presence of these three mucins has been verified at both the mRNA and protein levels, using Northern blot analysis or PCR, in situ hybridization, immunohistochemistry, and immunoblot analysis. Multiple methods are necessary for verification of expression of mucins, first, because low levels of mucin mRNA may not be translated, and because nonspecific binding of proteins (e.g., antibodies) to mucins is well known. The measure of relative RNA levels of the three membrane-associated mucins in conjunctival samples by real-time PCR suggest that MUC1 and MUC16 transcripts are the lowest in number, as compared to MUC4 mRNA levels.[4] Difficulty in obtaining human corneal epithelial samples has prevented similar comparisons for corneal epithelium.

In situ hybridization has demonstrated that message for MUC1 is dispersed throughout the corneal and conjunctival epithelia, but immunolocalization studies demonstrate that the protein is present only in the apical cell membranes.[45] In conjunctival epithelium, the mucin appears to be present on apical as well as subapical cells. In both epithelia, detection of the mucin with monoclonal antibodies specific to MUC1 is enhanced by pretreatment of tissue sections with neuraminidase to remove terminal sugars on the mucin.[45]

Unlike MUC1, there is regional variation of expression of mRNA for MUC4 across the ocular surface epithelia. In sections in which conjunctiva, limbus, and peripheral cornea are all present (Figure 4), message levels appear the greatest in the conjunctival and limbal epithelia, with a noted decrease in peripheral cornea and a greater diminution toward central cornea. Assay of levels of MUC4 message in central corneal epithelium by Northern blot also demonstrates lower message levels of MUC4 in central compared to peripheral corneal epithelium.[47]

Like the message distribution for MUC1, MUC4 mRNA appears in all cell layers in conjunctival and peripheral cornea, but there appears to be a greater intensity of S^{35}-labeled MUC4 probe in apical cells.[48] Immunolocalization of MUC4 in the conjunctival and corneal epithelia does not show the discrete membrane pattern of localization that other membrane-associated mucins do. Antibody to ASGP1 (HA-1), which recognizes the carboxy terminal region of the α subunit of rMuc4, binds to the cytoplasm of the entire stratified epithelium (see Figure 8 in Pflugfelder et al, 2000[47]).

Recent data indicate that the stratified cells of the corneal and conjunctival epithelia express the recently cloned, membrane-associated mucin MUC16.[46] This mucin was cloned and sequenced as the result of a long, 20-year effort to characterize the ovarian tumor marker CA125.[27,39] In situ hybridization studies demonstrate MUC16 message is in apical cells, as well as some suprabasal cells in cornea and conjunctival epithelium (Figure 4). Immunolocalization studies using antibodies specific to the mucin demonstrate that the glycoprotein is localized along the apical surface of both cornea and conjunctiva, in the position of the glycocalyx (Figure 4). MUC16 carries a carbohydrate epitope recognized by a monoclonal antibody designated H185 (see Argüeso et al, 2003[46]), and by immunoelectron microscopy, H185 localizes to the tips of the microplicae, in the position of the glycocalyx (Figure 5). The distribution of this antibody is altered on the ocular surface of patients with non-Sjogren dry eye (see below and Danjo et al, 1998[49]).

Assay for other membrane-associated mucins by PCR methods has demonstrated low levels of MUC11 mRNA (Gipson and Argüeso, unpublished data), but neither immunolocalization nor in situ hybridization studies have verified the presence of the mucin to date. PCR assays for MUCs 3, 12, 13 and 17 in corneal and conjunctival epithelia to date have been negative (Gipson et al, unpublished data).

Evidence exists that the extracellular domains of each of the membrane-associated mucins MUCs 1, 4, and 16 are shed into the tear film. Pflugfelder et al report detection of both ASGP1(α subunit) and ASGP2 (β subunit) in tears.[47] In several preliminary studies, all three of the

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mucins have been detected in tears (Spurr-Michaud et al and Hori et al, submitted to ARVO, 2004).

C. Lacrimal Glands and Accessory Lacrimal Glands as Sources of Mucin

Recent studies by Jumblatt et al[15] have demonstrated MUC7 mRNA and protein in four of six specimens of human lacrimal tissue RNA. Their in situ hybridization studies have demonstrated presence of message in some of the acinar cells of the gland. Protein for the small soluble mucin has also been detected in cellular extracts of the tissue by immunoblot, but curiously, the mucin has not been detected in tear samples assayed by immunoblot.[15] PCR analysis performed in the same study has identified message for MUCs 1 and 4, and also identified message for MUC5B in one sample, but in situ hybridization studies localizing the message of these mucins have not been done. rMuc4 has also been reported to be produced by rat lacrimal gland in both membrane and soluble forms.[50] To date, the mucins of the lacrimal gland appear to be either the small soluble mucins or membrane-associated mucins.

Accessory lacrimal glands, the glands of Kiaus and Wolfring, have cellular characteristics consistent with mucin secretion. Histologically, they appear to be a mixed population of cells, with both serous and mucus types of secretory vesicles—an appearance much like that of other submucosal glands.[51] These glands are, in all probability, producing mucins but, to date, they have not been examined for mucin production.

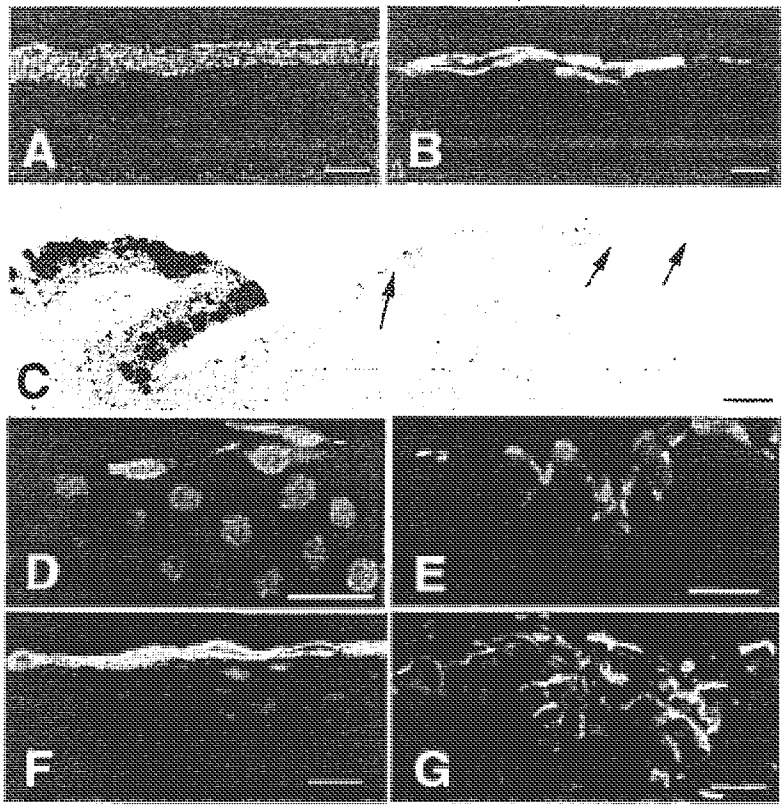

Figure 4. A and B demonstrate message and protein localization, respectively, of MUC1 in human corneal epithelium. C shows that mRNA localization of MUC4 diminishes from conjunctiva on the left, across the limbus (single arrow) toward the cornea on the right (two arrows). D and E show fluorescence in situ hybridization of MUC16 mRNA in cornea and conjunctiva, respectively, and F and G show immunohistochemical localization of MUC16 protein in human cornea and conjunctiva, respectively. Bars in A,C = 100 μm; Bars in B,D-G = 10 μm. (Reprinted from Gipson and Argüeso[4] with permission of Int Rev Cytol.)

D. Relationship of Ocular Surface Epithelial Mucins to the Tear Film

Of all the wet-surfaced epithelia of the body, the ocular surface is the most accessible, and, thus, this fluid-epithelial interface can be readily observed and studied. In spite of accessibility, the character, thickness, and distribution of mucins in the fluid are debated. The tear fluid-cell membrane interface over the apical cells of the guinea pig conjunctiva, as captured by rapid freeze and electron microscopy, is demonstrated in Figure 1C. The apical membrane folds or the microplicae of the apical cells of both cornea and conjunctiva have at their tips an electron-dense zone that interfaces with the tear film components.[52] This electron-dense zone, or glycocalyx, has in it the extracellular domains of the membrane-associated mucins expressed by the apical cells. Upon high magnification, striations within the glycocalyx can be seen (Figure 1C), and each probably represents individual, highly glycosylated, membrane-associated mucins, which are estimated to extend 200–500 nm from the cell membrane.[16] Nichols' high resolution electron microscopy also shows that actin cytoskeletal filaments insert into the tips of the microplicae and extend into the cytoplasm (Figure 1C).[52] Perhaps these actin filaments are the site of interaction with the cytoplasmic domain of the membrane-associated mucins.[23] Localization of the monoclonal antibody H185, which recognizes a carbohydrate epitope carried by the membrane-associated mucin MUC16, by immunoelectron microscopy,[46] shows labeling on the microplicae of human corneal and conjunctival epithelia, both in vivo and in vitro (Figure 5A and B).[53] These data verify the presence of the membrane-associated mucin in the glycocalyx.

The granular material above the glycocalyx in Figure 1C is assumed to be the gel-forming mucin secreted by the conjunctival goblet cells, which, in its hydrophilic extended network, houses other bactericidal proteins and

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fluids secreted by the lacrimal and accessory lacrimal glands. A diagram depicting this arrangement is shown in Figure 5C. Early diagrams depict a three-layered tear film with a sharp mucus-aqueous layer interface, but, since mucins are highly hydrophilic and some are small and soluble, it is more likely that mucins mix throughout the aqueous phase of the tear film (Figure 5C). Studies using interferometry, as well as confocal microscopy, have suggested that the mucus component is thicker than once envisioned, with measurements ranging from 3–30 μm.[54,55] Perhaps these methods include the microplicae with their associated glycocalyx layer composed of membrane-associated mucins that may extend 1–3 μm into the tear film. Also, since the mucus secreted from the goblet cell moves around over the surface of the eye and is cleared by blinking, that component of the tear film may vary, depending on the area of the palpebral fissure.

Secreted mucus may be envisioned as the cleanup or "janitorial service" of the ocular surface; the fully hydrated mucin multimers move over the glycocalyx, wrapping up dirt, debris, and unwanted pathogens, then moves them off the eye through the nasolacrimal duct. In order to function as the cleanup, particle-moving blanket that mucus layers of all wet-surfaced epithelia provide, the layer must move freely over the surface. The eyelids, during the blink, facilitate this process, moving the mucus layer and tear film toward the exiting nasolacrimal duct. The movement of the mucus layer over the glycocalyx implies that the mucins of the membrane-associated type are disadhesive, preventing strong interactions with the secreted mucins. Indeed, as stated earlier, experimental data indicate that the glycosylated tandem repeats of the membrane-associated mucins have disadhesive properties.[22]

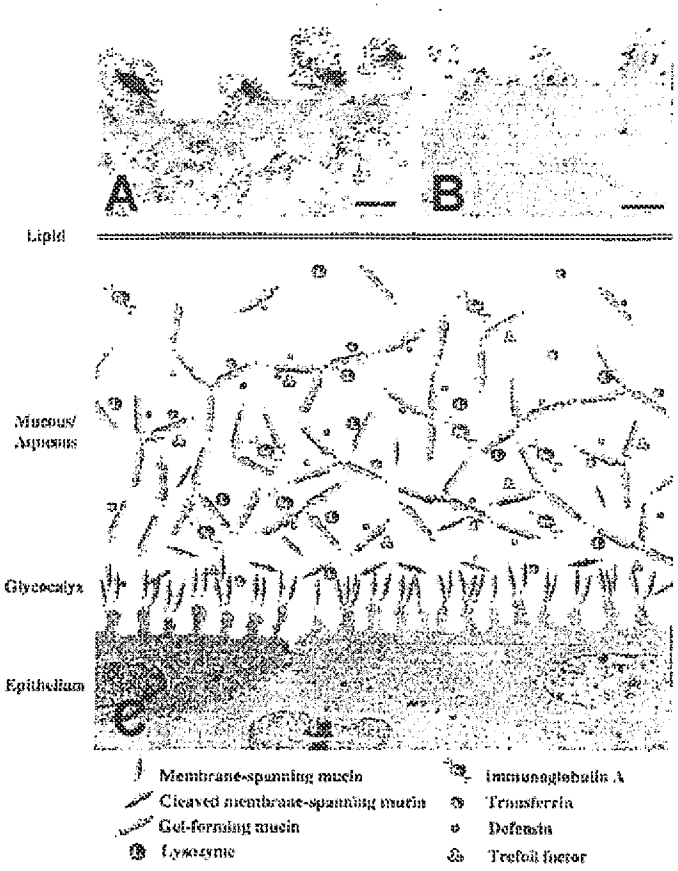

Figure 5. Immunoelectron microscopic localization of MUC16 on microplicae of cultured human corneal epithelial cells (A) and native corneal epithelial cells (B). Data shown in A and B support the diagram (C) of tear fluid composition and its association with membrane-associated mucins at the tips of the microplicae. The diagram shows MUC5AC multimers in solution in the tear fluid, along with other protective molecules secreted by the lacrimal gland. Negative charges on the mucins prevent their direct association and allow movement of goblet-cell-derived MUC5AC over the surface of the eye for its janitorial duties of collecting and removing debris and harmful pathogens. (Reprinted from Gipson and Argüeso[6] with permission of Int Rev Cytol.)

Cells that first appear at the surface of the stratified cornea, epithelium during normal cell differentiation and turnover have numerous microplicae and, over time, as the cell ages, these membrane projections disappear.[56] Antibodies to carbohydrate epitopes on membrane-associated mucins bind avidly to the newly emerged cells and their microplicae, and as the number of microplicae decreases on cells, so does the amount of antibody binding.[57] These data suggest that shedding of mucins occurs over time from the cell surface, leaving the oldest cells without both microplicae and membrane-associated mucins. The oldest cells may lose their disadhesive character and, thus, adhere to the mucus of the tear film to be removed and disposed of by mucus entrapment and removal through the nasolacrimal duct. These data also suggest that the microplicae structure is held in place by the membrane-associated mucin-actin cytoskeleton association.

IV. METHODS OF ASSAY OF MUCINS IN NORMAL SUBJECTS AND DRY EYE PATIENTS

In the past, the major limitations to the assay of human ocular mucins from tears or tissues have been the small amount retrievable from the ocular surface and the lack of appropriate biochemical tools to specifically identify individual mucins. Several analytical techniques (i.e., real time PCR, immunoassay) with high sensitivity and with a high specificity are now available for mucin analysis. These techniques have been successfully used to identify, localize, and quantify individual mucin mRNAs and proteins in samples collected from normal subjects and from patients with dry eye. This section summarizes recent research in the analysis of mucins collected by different methods from the ocular surface.

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A. Impression Cytology for Mucin Mrna and Protein Analysis

Impression cytology is a simple, inexpensive, and noninvasive technique to collect epithelial cells from the ocular surface.[58] After instillation of a topical anesthetic into the surface of the eye, a sterile impression disc (e.g., nitrocellulose, polyethersulfone) is applied on the bulbar conjunctiva for a short period of time and then carefully removed.[59,60] This procedure allows the collection of apical and subapical cells of the conjunctival epithelium, as well as goblet cells. The method is particularly useful for the analysis of mucins, as membrane-associated and gel-forming mucins are present in the suprabasal cells of the conjunctival epithelium and in the goblet cells, respectively. Conjunctival epithelial cells collected from a single individual can then be used to analyze mucin mRNA expression and mucin protein.

Recent progress in cloning and characterization of mucin genes has facilitated the use of PCR to determine the mucin mRNA repertoire in cells collected by impression cytology. Because of the small amount of starting material, it is necessary to pool the nitrocellulose discs collected from both eyes of the subject. RNA isolation from the discs, reverse-tranciption into cDNA, and real time-PCR analysis of the mucin mRNA content can be determined as described previously.[42] Using this method, the membrane-associated mucins MUCs 1, 4, and 16, as well as the gel-forming mucin MUC5AC, were found in a population of 18 normal individuals.[62] The relative levels of MUC1 and MUC16 transcripts were the lowest in number, as compared to MUC4 mRNA levels.[4] In addition, MUC7 mRNA and low levels of MUC2 mRNA have also been found using conventional PCR in conjunctival cells collected by impression cytology[61]; however, it is not known whether these transcripts are translated.

Samples collected by impression cytology are also suitable for mucin protein analysis by immunofluorescence[47,59,62] and flow cytometry.[60] Immunofluorescence microscopy has been used to detect MUC4 in impression cytology discs collected from human cornea and conjunctival epithelia,[47] as well as the H185 carbohydrate epitope on MUC16 from conjunctival epithelium.[49] MUC5AC has been detected by flow cytometry in goblet cells from samples of conjunctival epithelium collected by impression cytology.[60]

B. Tear Collection for Elisa and Immunoblot Analysis of Mucins

The gel-forming mucin MUC5AC has been detected in tears from normal individuals, collected by micropipette or after extraction from Schirmer strips.[42,63] In these studies, the relative amount of MUC5AC mucin was evaluated by ELISA or by direct immunoassay on the Schirmer strip, using polyclonal antibodies raised against synthetic peptides corresponding to nonglycosylated domains flanking the central tandem repeats in the MUC5AC molecule. The results showed that, when the tear samples were not pretreated with glycosidases, the amount of MUC5AC was highly variable between individuals. Interestingly, despite the fact that the MUC5AC antibodies were against nonglycosylated regions, treatment with neuraminidase—a glycosidase that removes terminal sialic acid on glycoproteins—increased the binding of the antibody to the mucin, decreasing the variability between individuals.[42] This result suggests that differential mucin glycosylation in some individuals masks recognition of the protein epitope recognized by the MUC5AC antibody. In semi-quantitative studies, it is then useful to partially deglycosylate the sample to facilitate access of the antibody to the mucin apoprotein.

The amount of membrane-associated mucin shed into the tear film by the ocular surface epithelia can be assessed by immunoblot. Pflugfelder et al have detected the two MUC4 subunits (ASGP1/α subunit and ASGP2/β subunit) in tears collected with porous polyester rods from normal volunteers, using immunoprecipitation techniques followed by SDS-PAGE and immunoblot analysis.[47] In addition to MUC4, the presence of MUC1 in tears has also been detected by immunoblot analysis of Schirmer strips of human tear fluid.[64] Surprisingly, despite the fact that both RNA and protein corresponding to the secreted MUC7 mucin are present in the normal human lacrimal gland and conjunctival tissue, MUC7 protein has not been detected in tears by immunoblot.[15] Perhaps the concentration of MUC7 in lacrimal tears is too low to be detected with the colorimetric immunoblot technique.

C. Biopsy of Conjunctiva for Immunolocalization and In Situ Hybridization

Collection of conjunctival biopsies from living donors for mucin analysis is a much more invasive method than impression cytology; however, it allows localization of the specific sites of mucin mRNA synthesis and protein expression through all the cell layers of the epithelium. Biopsies are obtained from the bulbar conjunctiva of patients who are undergoing cataract surgery. Distribution of mucin mRNA and protein is then analyzed by ISH and immunological techniques, respectively. It is always advisable to use a combination of ISH and immunohistochemistry to demonstrate mucin distribution, since detection of mucins exclusively by immunohistochemistry may be subject to error due to poor characterization of the mucin antibodies and to the "sticky" nature of mucins, which induces nonspecific binding.[65]

For ISH, the biopsy is normally fixed in 4% paraformaldehyde and embedded in paraffin.[48] The presence of tandemly repeated sequences in the nucleotide sequence of mucins has facilitated their analysis by ISH, since probes to the tandem repeat bind at multiple sites along the mucin mRNA, enhancing the signal and facilitating mucin mRNA detection.[48] The method is not quantitative, since mucin genes exhibit polymorphisms in the tandem repeat sequence.[48] The presence and distribution of MUC1 and 16 transcripts have been demonstrated by ISH in corneal

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and conjunctival epithelia.[45,46] MUC4 mRNA has been demonstrated in the stratified conjunctival and limbal epithelial cells but not central cornea, whereas MUC5AC transcripts are present in conjunctival goblet cells.[8] MUC7 mRNA has been detected by ISH exclusively in the lacrimal gland.[15] For immunohistochemistry, the biopsy can be frozen, or embedded in paraffin, depending on the antibody. Antigen retrieval techniques, such as microwave, proteolytic digestion, or deglycosylation may be required to unmask the epitope recognized by the antibody.[66]

V. ALTERATION OF MUCINS IN DRY EYE

Only with the recent development of methodologies has it become possible to measure specific mucins in ocular surface epithelia and tears of patients with drying ocular surface disease. The few studies that have been done indicate alteration in secreted mucin mRNA and protein, as well as alteration in distribution and/or glycosylation of membrane-associated mucins.

It has long been known that a decrease in the density of mucin-producing goblet cells in the conjunctival epithelium is a common characteristic in dry eye patients.[62,67,69] As the disease progresses in severity, the number of goblet cells decreases further, and squamous metaplasia and keratinization of the ocular surface ensues. This led to speculation that mucus deficiency could be the direct cause of tear film instability.[70,71] Initial studies measuring hexosamine and O-linked oligosaccharides as indicators of mucin found a correlation between the content of mucin-like glycoprotein in tears and goblet cell density in conjunctiva.[70,71] Surprisingly, the data also showed a substantial amount of mucin-like glycoprotein present, even when only few goblet cells were present.[70,71] Perhaps this mucin-like glycoprotein is shed membrane-associated mucin, now measurable in tear fluid.

A. Decreased Levels of Goblet-cell-associated Mucin MucSac in Dry Eye

Recent data using real-time RT-PCR have shown that the number of RNA transcripts for MUC5AC in the conjunctival epithelium of patients with Sjogren syndrome was significantly lower than in normal individuals.[42] As measured by ELISA, the protein levels of MUC5AC were also significantly reduced in the tear fluid of the same patients, corroborating mRNA data obtained using real-time RT-PCR (Figure 6). Additional evidence correlating a reduction in MUC5AC mucin with dry eye is the reduction in the percentage of MUC5AC-positive conjunctival cells in dry eye patients, as determined by flow cytometry.[60]

Tei et al have correlated a reduction in MUC5AC mucin gene expression with the goblet cell density, using an animal model of ocular surface keratinization, using vitamin A-deficient rats.[72] In this study, the number of rMuc5AC transcripts in the ocular surface epithelium of rats deprived of vitamin A for 20 weeks decreases, as did identifiable goblet cells. The possibility that another secreted mucin increases remains a hypothesis to be tested.

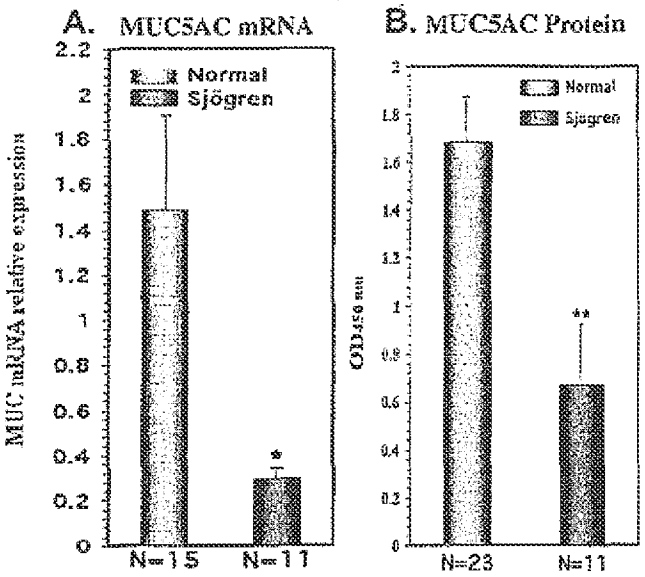

Figure 6. Levels of MUC5AC mRNA (A) and protein (B) were significantly less in Sjogren patients (N = 11) as compared to normal subjects (N = 15-30) (Reprinted from Argüeso et al[42] with permission of Invest Ophthalmol Vis Sci.)

These studies provide definitive evidence that the levels of mRNA and protein of the gel-forming mucin MUC5AC decreases in dry eye, correlating with a reduction in goblet cell density. Measurement of MUC5AC mucin in tears is a valuable noninvasive method to detect dry eye disorders and could potentially be used to evaluate the effect of pharmaceutical products in the treatment of dry eye.

B. Alterations of Membrane-associated Mucins in Dry Eye

In addition to the recent studies demonstrating alteration in the amount of the secreted mucin MUC5AC in Sjogren dry eye,[42] membrane-associated mucins are altered in non-Sjogren dry eye.[49] Danjo et al demonstrated a significant difference in the binding pattern of an antibody against a carbonate epitope antibody (H185) carried by MUC16 to conjunctival epithelium obtained by impression cytology in normal eyes compared with those of patients with non-Sjogren dry eye.[49] In normal eyes, the antibody bound to apical cells in a mosaic pattern that shows various degrees of H185 binding to cells, as determined by immunofluorescence microscopy on impression cytology samples (Figure 7). The mosaic pattern was absent in patients with non-Sjogren dry eye. These data were corroborated by immunoelectron microscopy (Figure 7B and D). The question arises—is the alteration in distribution of H185 antibody binding a result of lack of expression of the membrane-associated mucins or in their glyco-

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sylation? We previously reported that the levels of MUC1 mRNA from conjunctival impression cytology showed a trend toward decreasing amounts in patients with Sjogren syndrome compared with norma. subjects, but there was no statistically significant difference.[42] Additionally, there was no difference in levels of MUC4 mRNA. A thorough study of the level of all the membrane-associated mucin protein in tears has not been done. Pflugfelder e. al, using an antibody recognizing an epithelial membrane-associated mucin expressed in human conjunctiva (AMEM2), found less binding to the conjunctiva in patients with Sjogren syndrome than in normal subjects, as determined by immunohistochemistry analysis.[62]

Two pieces of data suggest that the alteration in H185 mucin distribution in dry eye is related to glycosylation in the membrane-associated mucins. Firstly, there is a loss of binding of the H185 antibody to apical cells of conjunctival epithelium in non-Sjogren dry eye. Secondly, in more advanced severe dry eye, such as in ocular cicatricial pemphigoid (OCP), there is alteration in distribution of glycosyl transferases tha: glycosylate mucins in the conjunctival epithelium, with complete loss of their expression in keratinized regions. Further investigation is needed to determine the level of membrane-associated mucins in tears of dry eye patients and factors affecting their expression.

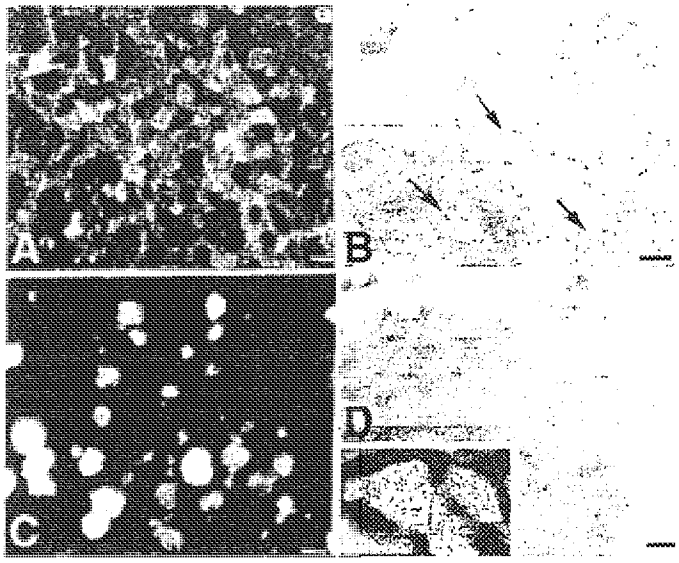

Figure 7. Distribution of the H185 carbohydrate epitope carried by MUC16 is altered in non-Sjogren dry eye. The pattern of distribution in normal subjects is shown by immunofluorescence microscopy (A) and immunoelectron microscopy (B) of samples taken by impression cytology. Note cobblestone pattern of distribution of apical cells in A and apical and cytoplasmic immunogold particles in B. Altered distribution of binding of H185 antibody in non-Sjogren dry eye samples is demonstrated by immunofluorescence microscopy (C) and immunoelectron microscopy (D). Note less of binding to apical cells in C, but enhanced bright spots of binding to goblet cells. Immunoelectron microscopy corroborates loss of binding to apical cells (D), but dense immunogold labeling to mucin packets (inset). Bars in A,C = 20 μm; Bars in B,D = 0.5 μm. (Reprinted from Gipson and Argüeso[4] with permission of Int Rev Cytol.)

C. Glycosylation of Mucins: Alterations in Dry Eye

The importance of studying mucin glycosylation arises from the high numbers of clustered O-glycans on mucins, alterations of which may affect the physiology of mucins or mucous epithelia. Glycosyltransferases are the enzymes that transfer monosaccharide residues from their activated forms, the sugar nucleotides, to growing oligosaccharide chains on mucins. Glycosyltransferase efficiency is dictated by availability of donor sugars, which, in turn, is a function of tissue metabolic status and, therefore, removed from direct mucin genetic control.

Mucin O-glycosylation is initiated post-translationally by the enzymatic addition of N-acetylgalactosamine (GalNAc) to serine and threonine residues (Figure 8A). The family of enzymes that catalyze this initial step, UDP-GalNAc:polypeptide N-acetylgalactosaminyl transferases (GalNAc-Ts), regulates the density and the position of O-linked sugar chains in the protein backbone.[73] In the stratified epithelium of the normal human ocular surface, the distribution of members of the GalNAc-transferase family is cell-layer and cell-type specific (Figure 8B).[74]

The highly ordered distribution of GalNAc-transferases observed in the conjunctiva of normal individuals is altered during the keratinization process, which may lead to altered glycosylation of mucins and severe ocular surface dryness, such as occurs in OCP. There is increased GalNAc-transferase expression in the nonkeratinized conjunctival epithelium of OCP patients, suggesting that there is an initial attempt by the epithelium to maintain a wet-surfaced phenotype by upregulating or modifying mucin-type O-glycosylation. A marked reduction in GalNAc-transferase antibody binding was observed in the late-stage, keratinized conjunctival epithelia of OCP patients, which may result in the aberrant synthesis of mucin O-glycans and, thus, in an alteration of the physicochemical properties of mucins.

The biosynthesis of O-glycan structures following polypeptide GalNAc-transferase action is controlled by the repertoire of glycosyltransferases expressed by the cell, their level of activity, and their spatial organization over the Golgi apparatus.[75] Elongation of the Tn antigen, as the mucin passes through the Golgi, generates up to eight different glycan core structures, the most common being core 1 and core 2 (Figure 8A).[76] These core structures can then be extended by addition of polylactosamine and/or terminated by addition of one of a large repertoire of terminal carbohydrates (see example in Figure 8A). These extensions and terminations occur in a tissue- and cell-type-specific manner. Although there are no structural studies demonstrating the carbohydrate configuration of specific ocular mucins, it has been proposed that they contain mainly short

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oligosaccharide chains, such as Tn (GalNAcα1Ser/Thr) or sialyl Tn antigens (NeuAcα2-6GalNAcα1Ser/Thr) attached to the protein backbone.[77] Other terminal carbohydrate-related epitopes, such as the blood group ABO and Lewis antigens (Figure 8A), have also been found within the goblet cell mucin packets and epithelial cells, indicating that mucin carbohydrate architecture may be more complex. Little is known regarding terminal sugars on ocular surface mucins, nor is it known whether each mucin type has unique carbohydrate characteristics.

Several studies have demonstrated an altered expression of carbohydrates in dry eye syndrome.[59,78] Versura et al. using a lectin-colloidal gold technique in combination with transmission electron microscopy, found alterations in the carbohydrate content of glycoconjugates of conjunctival goblet cells in dry eye patients as compared to normal subjects.[78] In tears, Garcher et al have shown that a sialylated Lewis A carbohydrate epitope on glycoconjugates/mucins was significantly decreased in patients with dry eye syndrome.[79] As stated above, immunohistochemical studies have also reported an alteration in the distribution of H185 mucin carbohydrate epitope carried on MUC16 in dry eye syndrome patients as compared to normal individuals (see below and Danjo et al, 1998[59]). Although these data provide evidence of changes in mucin O-glycosylation in dry eye, the O-glycan composition of each mucin at the ocular surface is not known, nor is it known how the glycosylation of each individual mucin gene product is affected in dry eye.

VI. DRY EYE THERAPEUTICS AND THEIR EFFECT ON MUCIN PRODUCTION/ DISTRIBUTION

The main therapy for dry eye is application of artificial tears. Recently, however, several drugs/agents have been reported to induce mucin expression or secretion by the human ocular surface epithelia. These may be good candidates as alternatives to artificial tears for treatment of dry eye, especially for the mucin-deficient type of dry eye. Recent data indicate that some of these drugs/agents regulate secreted mucins, while others regulate membrane-associated mucins. Since mucins are differentially regulated, perhaps application of reagents that affect both mucin types will be additive in their efficacy.

A. Cyclosporine A

Recent studies suggest that dry eye syndrome is a disease associated with inflammation.[60,80-83] For example, a significant increase in expression of the inflammatory markers, human lymphocyte antigen-DR (**HLA-DR**) and intercellular adhesion molecule-1 (**ICAM-1**) by conjunctival epithelial cells, was found in patients with dry eye syndrome.[60,80] Cyclosporine A is an immunosuppressive agent commonly used systemically to treat inflammatory diseases, such as psoriasis or rheumatoid arthritis.[84] Topical cyclosporine A has been used to treat corneal transplant rejection[85] and Mooren's ulcer.[86] Recently, topical

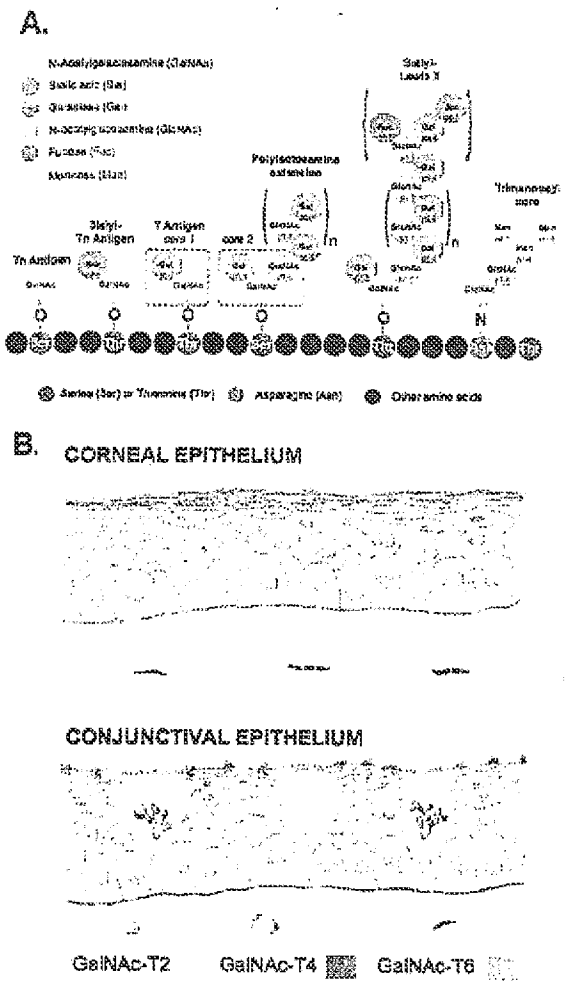

Figure 8. A. Glycans commonly found attached to mucins. The biosynthesis of O-linked oligosaccharides or O-glycans is initiated by the enzymatic addition of N-acetylgalactosamine to serine or threonine residues to form the Tn antigen. Subsequent modification of the Tn antigen by addition of carbohydrate generates a variety of different configurations, such as sialyl-Tn antigen and the T antigen (Core 1). N-Acetylglucosamine can be linked to the T antigen to form the Core 2 structure, which can be further elongated by the sequential addition of lactosamine residues. A large number of terminal structures, such as the blood group ABH and Lewis antigens (e.g., sialyl-Lewis X), have been found on mucin oligosaccharide chains. Minor amounts of N-glycans containing the Trimannosyl core are also present on mucins.[2] B. Diagram summarizing cell layer and goblet-cell-specific distribution of GalNAc transferases in human corneal and conjunctival epithelia. (Reprinted from Gipson and Argüeso[4] with permission of Int Rev Cytol.)

cyclosporine A emulsion (RESTASIS™, Allergan Inc.; Irvine, CA) received approval from the FDA for patients with dry eye. Topical cyclosporine A treatment of dry eye patients has been reported to be clinically effective[87,88] by reducing the presence of inflammatory markers in conjunctiva (HLA-DR and CD40).[82] Kunert et al demonstrated that 6-month treatment with topical cyclosporine A (0.05%) not only reduced the number of cells expressing

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the lymphocyte activation markers CD11a and HLA-DR,[89] but also increased the number of goblet cells in the conjunctival biopsy of patients with dry eye syndrome.[69] Reducing inflammation of the ocular surface may lead to reconstitution of healthy conjunctival epithelial cells, resulting in an increase in the number of conjunctival goblet cells and a concomitant increase in secreted mucin MUC5AC in tears.

B. P2Y2 Agonist

The P2Y2 receptor is a nucleotide receptor that is localized on diverse cell types, including epithelial, neuronal, glial, bone, and endothelial cells.[90] At the ocular surface, the P2Y2 receptor gene is expressed by corneal and conjunctival epithelium of both rabbit and monkey, as determined by in situ hybridization.[91] Jumblatt et al demonstrated that the exogenous P2Y2 nucleotide receptor agonists, adenosine 5'-triphosphate (**ATP**) and uridine 5'-triphosphate (**UTP**) stimulated mucin secretion in human and rabbit conjunctiva, as measured by lectin binding.[92] The mechanism of this regulation is not completely understood; however, the ATP- or UTP-associated induction of mucin-like secretions in the rat tracheal goblet cell line SPOC1 is reported to be regulated by Ca^{2+} and protein kinase C-dependent pathways.[93] Recently, a more stable P2Y2 receptor agonist, INS365 (Inspire Pharmaceuticals, Inc.; Durham, NC), was reported to stimulate tear secretion in a rat dry eye model.[94] This drug has been favorably evaluated in clinical trials (source: Inspire Pharmaceuticals, Inc) and is being developed for distribution by Allergan, Inc. It is not clear whether the drug transiently affects goblet cell MUC5AC expression, or whether it affects shedding of the membrane-associated mucins from the ocular surface.

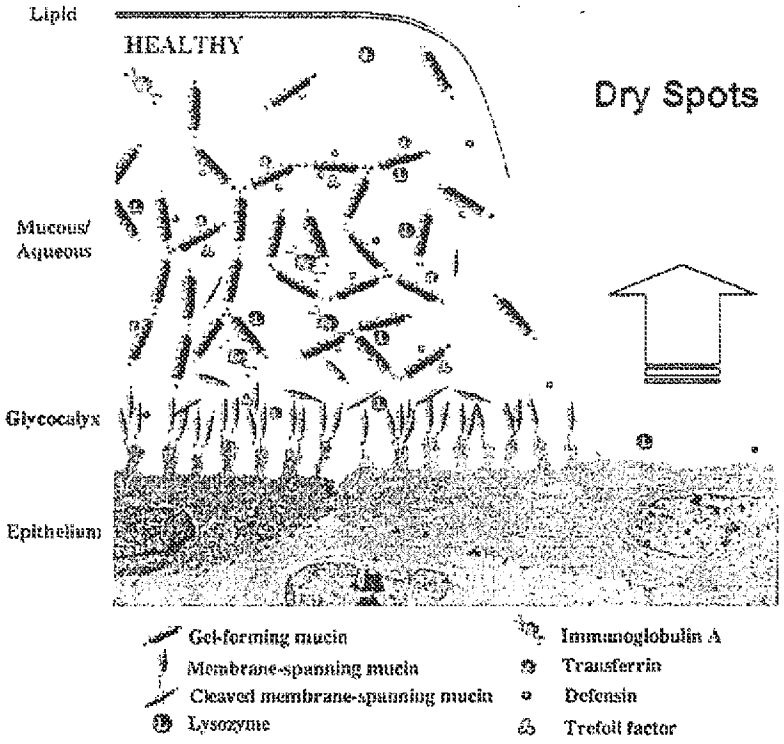

Figure 9. Diagram depicting an hypothesis that loss of hydrophilic secreted and membrane-associated mucins leads to loss of hydration and, in turn, to the formation of dry spots on apical cells of the ocular surface. Loss of the hydrophilic molecules leads to loss retention of fluid, which, even with functional lacrimal glands, could lead to lower Schirmer values. (Reprinted from Argüeso and Gipson[43] with permission of Exp Eye Res.)

C. 15-(S)-HETE

The eicosanoid, 15-(S)-hydroxy-5,8,11,13-eicosatetraenoic acid (**HETE**),[95] is a metabolite of arachidonic acid by the lipoxygenase pathway. Previously, 15-(S)-HETE was known to stimulate secretion of mucus by airway epithelium.[96,97] Jackson et al reported that topical application of 15-(S)-HETE to the rabbit ocular surface increased the thickness of the mucin layer on the surface of the corneal epithelium within 5 minutes, as measured by image analysis of electron micrographs.[95] Furthermore, Jumblatt et al demonstrated that 15-(S)-HETE increased the amount of MUC1 protein, but not MUCs 2, 4, 5AC, or 7 in human conjunctiva, as determined by dot-blot assay.[64,98] These data suggest that 15-(S)-HETE may upregulate the expression of some of the membrane-associated mucins, but not the secreted mucins. The expression levels have not been measured; thus, it is not known if the reagent induces an increased release of MUC1 from cells or whether there is an increase in production or shedding of the mucin. It has been hypothesized that the change in MUC1 levels may be associated with the protein kinase C signal transduction pathway.[95]

D. Gefarnate

Gefarnate (3,7-dimethyl-2,6-octadienyl-5,9,13-trimethyl-4,8,12-tetradecatrienoate) is widely used for patients with gastric ulcer and gastritis. The mechanism of action of gefarnate in the stomach is to normalize mucous secretion, which restores the defensive mucous barrier (as reviewed in Nakamura et al[99]). They reported that 1% gefarnate eye drops increased presence of mucin-like glycoproteins in media of cultured rat cornea, and, in an in vivo study, the drug reduced dessication in rabbit cornea[100] allowed to dry by preventing eyelid closing with a speculum. Furthermore, treatment with the drug for 7 days increased PAS-positive cell (goblet cell) density in rabbit conjunctiva.[99] Toshida et al demonstrated that treating a mild alkali injury to conjunctiva in a monkey model with gefarnate for 4 weeks increased the number of goblet cells

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in the conjunctiva, as well as the amount of MucSAC in tears.[101] The rat corneal culture data suggest that gefarnate may induce shedding or expression of membrane-associated mucins, and the in vivo data suggest that goblet cell secretion and differentiation are also induced.

E. Corticosteroids

Anti-inflammatory therapy using topical corticosteroids has recently been reported to be an efficacious therapy for patients with dry eye syndrome.[102,103] Marsh et al reported the efficacy of topical administration of 1% nonpreserved methylprednisolone for patients with severe conjunctivitis sicca, demonstrating relief from irritation, a decrease in fluorescein staining, and resolution of filamentary keratitis.[102,103] Avunduk et al compared treatment of KCS patients with the corticosteroid fluorometholone (**FML**) to treatment with the nonsteroidal anti-inflammatory flurbiprofin.[103] They found the corticosteroid to be more efficacious, producing the greatest decrease in symptom severity, fluorescein and rose bengal staining, increase in goblet cell numbers, and decrease in HLA-DR-positive cells. In terms of the effect of corticosteroids on mucin expression, membrane-associated mucin MUC1 mRNA is dramatically upregulated by a 24-hour incubation of immortalized human conjunctival epithelial (HCjE) cells with dexamethasone (1 μM), as determined by quantitative real-time PCR.[104] Other mucin genes (4, 5AC, 16) were unaffected by dexamethasone. Perhaps upregulation of membrane-associated mucins lubricates the eye and leads to increased differentiation of goblet cells.

F. Autologous Serum

Two clinical reports describe the efficacy of applying autologous serum for the treatment of dry eye due to Sjogren syndrome.[105,106] Serum contains a number of growth factors, vitamin A, and anti-inflammatory factors that are needed for maintenance of a healthy ocular surface. We demonstrated that the expression of membrane-associated mucins MUCs 1, 4, and 16 were upregulated by serum in HCjE cells, both at the mRNA and protein level, as determined by quantitative real-time PCR and Western blot analysis, respectively.[107] We also found that the pattern of regulation of each membrane-associated mucin was different from the others, suggesting that the three mucins are independently regulated. These data suggest that the efficacy of autologous serum application for dry eye may be related to the upregulation of expression of these three membrane-associated mucins in human ocular surface epithelia. Study of the alteration of the expression of secreted mucin has not been performed.

G. Vitamin A

It is well known that the ocular surface has an absolute requirement for vitamin A. Lack of sufficient vitamin A causes abnormal differentiation of the ocular surface, resulting in keratinization of both conjunctival and corneal epithelial cells.[108] Depletion of vitamin A in the diet of rats caused loss of expression of rMuc5AC and rMuc4 in ocular surface epithelia, whereas rMuc1 levels were unaffected.[72] Topical vitamin A has been reported to be effective as a treatment for severe squamous metaplasia,[109,110] but not for keratoconjunctivitis sicca.[110,111] We reported that treatment with 100 nM retinoic acid, the biologically active form of vitamin A, in the absence of serum upregulated the expression of both MUC4 and MUC16 mRNA and protein in HCjE cells, whereas MUC1 was unaffected.[107] These data may explain the efficacy of retinoic acid (vitamin A) for severe mucin-deficient squamous metaplasia.

VIII. SUMMARY: HYPOTHESIS REGARDING MUCIN ALTERATIONS IN DRY EYE

With the characterization of expression of mucins by the ocular surface epithelia, studies of their alteration in ocular disease have begun. These studies have required development of new techniques to assay changes in expression level of mucin genes, content of mucins in tears, and measurements of their post-translational modification. To date, decreases in levels of MUC5AC mRNA and tear protein have been demonstrated in patients with Sjogren syndrome. Glycosylation also seems to be altered on membrane-associated mucins; distribution of the H185 carbohydrate epitope on MUC16 is altered in non-Sjogren dry eye. Based on these early findings, as well as on new information that goblet cell mucin moves over an epithelial surface glycocalyx that is composed of membrane-associated mucins, which are especially prevalent in microplicae, we have developed an hypothesis regarding drying at the ocular surface. Decreased production of MUC5AC, as well as changes in membrane-associated mucins (either downregulation, altered glycosylation, or increased shedding) leads to loss of microplicae and less tear adherence to epithelial cells on localized regions of the ocular surface (Figure 9). These "dry spots" stain with rose bengal and lead to shorter tear breakup times. The loss of the highly hydrophilic mucin population implies that, even if lacrimal fluid is available, it will not be retained at the ocular surface.

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Ulcerative Colitis in Children
Medical Management

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Contents

Abstract

Ulcerative colitis is a chronic relapsing inflammatory disorder of the colonic mucosa of unknown etiology. The inflammatory process involves the mucosa and submucosa in a continuous segment of bowel with rectal involvement in almost all cases. Since its etiology is unknown, therapy is directed at modulating the inflammatory response in order to control symptoms and to prevent relapses.

5-aminosalicylates and corticosteroids have been the most widely used therapeutic agents for treatment of ulcerative colitis. Recently, experience has been gained with the use of other immunomodulators, such as mercaptopurine, azathioprine, methotrexate, cyclosporine, and tacrolimus, in pediatric patients. Colectomy is indicated in patients with severe colitis who do not respond to intensive medical therapy.

The care of children with ulcerative colitis not only involves control of symptoms from gastrointestinal and extraintestinal manifestations, but also optimizing growth and development. The complications of chronic inflammation and long-term medical therapy must be weighed against the risks and benefits of surgery for children and adolescents with this condition.

Ulcerative colitis was first described as an entity distinct from infectious colitis in 1875. Crohn et al.[1] described a transmural inflammatory disease of the small intestine distinct from ulcerative colitis in 1932. Diagnostic criteria distinguishing ulcerative colitis from Crohn's disease were established in 1960.[2] Due to similar clinical, endoscopic, and histologic features of ulcerative colitis and Crohn's disease, up to 12% of cases of inflammatory bowel disease (IBD) remain indeterminate.[3]

Ulcerative colitis occurs worldwide in patients of all ethnic backgrounds. Reported prevalence rates for ulcerative colitis range from 3.4/100 000 in Wales[4] to 7.5/100 000 in Sweden.[5] The incidence of ulcerative colitis increased in Wales from

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0.71/100 000 in 1983 to 1993,[4] to 1.7/100 000 in 1998 to 1999.[3] In Sweden, the incident rate increased from 1.4/100 000 in 1984 to 1986, to 3.2/100 000 in 1993 to 1995.[6] Data from the British Paediatric Surveillance Unit[3] indicate that children with IBD who have an Asian background are more likely to have ulcerative colitis (42%) than those of Caucasian or Afro-Caribbean backgrounds (28%).

1. Clinical Presentation

The age of onset of ulcerative colitis has a bimodal distribution, with peaks occurring during the second and third decades of life, and the fifth and sixth decades of life. The peak onset in pediatric patients occurs during adolescence.[7] Most cases of ulcerative colitis begin with diarrhea that is associated with rectal bleeding as the disease progresses. Fever, weight loss, abdominal pain, and tenesmus are usually absent in patients with mild colitis. Laboratory evaluation is often normal in patients with mild colitis, without anemia or hypoalbuminemia.

Determination of disease severity is used in guiding appropriate pharmacologic therapy. Patients with mild disease typically have less than four bowel movements daily, with passage of blood less than daily and no systemic symptoms. Patients with moderate colitis exhibit bloody diarrhea with five or more bowel movements daily, and fecal urgency, colicky pain, malaise, and low grade or intermittent fever. Abdominal tenderness, particularly in the left lower quadrant, is often present on physical examination. Laboratory evaluation usually reveals anemia, hypoalbuminemia, guaiac positive stool, and fecal leucocytes. Severe colitis is characterized by passage of more than six bloody stools per day, with abdominal tenderness, tachycardia, fever (temperature >38°C), weight loss, hematocrit <30%, and serum albumin <3 g/dl.

Laboratory findings in ulcerative colitis include fecal erythrocytes and leucocytes, anemia, and hypoalbuminemia. Nonspecific markers of inflammation including leucocytosis, thrombocytosis, elevated erythrocyte sedimentation rate, and elevated C-reactive protein concentration are frequently present. Electrolyte disturbances that may occur from dehydration due to the diarrhea associated with severe colitis may include hypokalemia, hypocalcemia, and hypomagnesemia. These electrolyte disturbances may contribute to colonic dysfunction, further complicating the clinical course.

Ulcerative colitis may present with extraintestinal manifestations that can precede symptoms of overt colitis. It can cause decreased linear growth velocity in children and adolescents secondary to the inflammatory process, although patients with Crohn's disease are twice as likely to have growth abnormalities as are those with ulcerative colitis.[8] Arthritis occurs in 2 to 15% of patients with ulcerative colitis.[9] A migrating polyarthritis or nondestructive arthritis of peripheral large joints may also precede the onset of gastrointestinal symptoms. Skin lesions associated with ulcerative colitis include erythema nodosum and pyoderma gangrenosum. The skin lesion of erythema nodosum typically erupts on the extensor surfaces of the arms and legs. Pyoderma gangrenosum is a progressive deep ulceration of the skin that often occurs in association with exacerbation of the gastrointestinal symptoms of ulcerative colitis.

2. Diagnostic Evaluation

Many other pediatric gastrointestinal disorders may mimic ulcerative colitis. The suggested diagnostic tests in table I can provide evidence to confirm the diagnosis of ulcerative colitis, and to screen for other conditions that can mimic ulcerative colitis. The symptoms of bacterial or parasitic gastroenteritis with pathogens such as *Salmonella, Shigella, Campylobacter, Aeromonas, Plesiomonas, Yersinia, Escherichia coli* O157:H7, *Clostridium difficile*, or *Entamoeba histolytica* may be indistinguishable from those of ulcerative colitis. The colonoscopic appearance of infectious and ulcerative colitis can be similar.[10] Since the results of stool cultures are negative in up to 50% of patients with presumed infectious self-limited colitis, attempts have been made to identify unique histologic findings that distinguish infectious colitis from IBD. However, the histologic features are often similar.[11] Acute inflammatory changes with cryptitis and crypt abscesses may be seen in infectious or ulcerative colitis. Chronic inflammatory infiltrates with lymphocytes and architectural distortion

Table I. Diagnostic testing for ulcerative colitis

Laboratory tests
Stool culture for bacterial pathogens
Stool ova and parasite examination
Stool for *Clostridium difficile* toxin
Stool for occult blood and leucocytes
Complete blood count
Serum chemistry profile including electrolytes, blood urea nitrogen, creatinine, total protein, albumin, and liver associated enzymes
Acute phase reactants: erythrocyte sedimentation rate, C-reactive protein
anti-Saccharomyces cerevisiae IgG and IgA antibodies
Perinuclear antineutrophil cytoplasmic antibodies
Radiographic tests
Upper gastrointestinal series with small bowel follow through
Endoscopic tests
Esophagogastroduodenoscopy
Colonoscopy

Ig = immunoglobulin.

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of the colonic mucosa with branched crypts or decreased goblet cells indicate ulcerative rather than infectious colitis.[11] Another association that makes distinguishing infectious from ulcerative colitis more difficult is that relapsing symptoms of ulcerative colitis may occur after successful eradication of organisms, causing acute bacterial gastroenteritis such as *Salmonella typhi*.[10]

Other causes of rectal bleeding in pediatric patients include Meckel's diverticulum, hemolytic-uremic syndrome, polyposis, Henoch-Schönlein purpura, and anal or rectal fissures. The rectal bleeding associated with Meckel's diverticulum or polyposis is usually painless and is not accompanied by fecal leucocytes. Hemolytic-uremic syndrome can be diagnosed by the combination of hemolytic anemia and elevated blood urea nitrogen. Rectal bleeding in Henoch-Schönlein purpura occurs in the context of the typical purpuric rash, arthritis, and renal findings. Fissures may be associated with constipation, or may be due to perianal complications of Crohn's disease, particularly if there is surrounding inflammation.

One goal in the diagnostic evaluation of children with IBD is to distinguish ulcerative colitis from Crohn's disease. Inflammatory changes in the colonic mucosa share similar features in these two diseases. Biopsy findings of transmural or granulomatous inflammation are pathognomonic of Crohn's disease, such that acquisition of multiple colonic biopsy specimens is important in the diagnostic evaluation. Since lower gastrointestinal tract findings may be indistinguishable in ulcerative colitis and Crohn's disease, diagnostic testing of pediatric IBD includes evaluation of the upper gastrointestinal tract with upper gastrointestinal contrast radiographs, and small bowel follow through and esophagogastroduodenoscopy. Upper gastrointestinal inflammation in IBD usually indicates Crohn's disease, but gastroduodenal inflammation has also been observed in children with ulcerative colitis.[12] Extensive small bowel inflammatory changes on contrast radiographs are associated with Crohn's disease.

Serologic markers may be helpful in distinguishing ulcerative colitis from Crohn's disease. Perinuclear antineutrophil cytoplasmic antibodies are detected in up to 83% of children with ulcerative colitis, but only 14 to 19% of children with Crohn's disease.[13] In contrast, anti-Saccharomyces cerevisiae IgG and IgA antibodies are more common in patients with Crohn's disease and are infrequent in ulcerative colitis. Thus, a combination of laboratory, radiographic and endoscopic tests is employed to differentiate between ulcerative colitis and Crohn's disease in pediatric IBD.

3. Etiology

Current theories speculate that infectious, environmental, and genetic factors may all contribute to the pathogenesis of ulcerative colitis, but the etiology remains unknown. The lesions of infectious colitis are similar to those of ulcerative colitis, although no infectious agent has been found as a cause.[14] Increased mucosal cytokines that indicate autoimmune processes contribute to the pathogenesis of IBD. Children with ulcerative colitis have higher levels of mRNA transcripts for interleukin (IL)-5 than children with Crohn's disease,[14] and the severity of ulcerative colitis is associated with a lower mucosal ratio of IL-1ra/IL-1β.[15]

4. Medical Management

The goals of medical management of ulcerative colitis are to control symptoms and prevent relapses. The pharmacologic therapy for ulcerative colitis involves the suppression of the inflammatory processes associated with this condition. The pharmacologic agent or combination of agents used is determined by the severity of the disease and the response to treatment. In addition to pharmacologic therapy, the consequences of ulcerative colitis on peer and family relationships, school attendance, and emotional well being should be included as part of the comprehensive management of this disorder. The medications that can be used in the treatment of ulcerative colitis are listed in table II. The role of specific medication in the management of ulcerative colitis is detailed in section 4.1 to section 4.7.

4.1 5-Aminosalicylates

4.1.1 Sulfasalazine

Sulfasalazine has long been recognized as effective treatment for mild to moderate ulcerative colitis. Sulfasalazine combines 5-aminosalicylate (5-ASA) and sulfapyridine, connected by an azo bond. The azo bond is cleaved by colonic flora. Sulfapyridine is absorbed and secreted in the urine, while 5-ASA is poorly absorbed and exerts an anti-inflammatory effect on the colonic mucosa. The mechanism of action is unknown, but it is speculated that 5-ASA inhibits production of arachidonic acid metabolites through both the cyclo-oxygenase and lipoxygenase pathways.

One study of ulcerative colitis reported remission of symptoms within 6 months of starting medical therapy in 90% of children with mild disease, and 81% of patients with moderate or severe disease.[16] After initial resolution of symptoms, 58% of patients with mild disease at presentation were asymptomatic at 1 year after diagnosis, while 40% experienced intermittent symptoms.[16] Patients with moderate to severe disease at the time of diagnosis had similar 1-year follow-up, with 45% in remission and 44% with intermittent symptoms.[16] Most patients (90%) with mild disease in this study responded solely to oral sulfasalazine or rectal or oral 5-ASA, although 27% required corticoster-

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Table II. Drugs used in the treatment of ulcerative colitis

Medication	Dosage	References
Sulfasalazine	50–60 mg/kg/day (max. 4g daily)	16
Mesalamine	30–60 mg/kg/day	17
Olsalazine	30 mg/kg/day	18
Balsalazide	Adult dose: 6.75g daily	19
Prednisone	1.0–2.0 mg/kg/day once or twice daily	20
Azathioprine	1.5–2.0 mg/kg/day	21
Mercaptopurine	1.0–1.5 mg/kg/day	21
Methotrexate	Initial dose 5 mg/wk, increase every 2–4 wks up to 20 mg/wk	22-24
Cyclosporine	4 mg/kg/day PO divided q12h	25,26
Tacrolimus	0.1 mg/kg/dose q12h	27
Infliximab	5 mg/kg/dose IV over 2h	28-30

h = hour; IV = intravenously; max = maximum; PO = orally; q12h = every 12 hours; wk = week.

ids within 12 months after diagnosis.[16] In contrast, 76% of patients with moderate to severe disease at presentation received orticosteroids during the first year of treatment.[16] Since sulfasalazine competitively inhibits folate absorption, folic acid supplementation is prescribed to prevent folic acid deficiency.

Although hypersensitivity reactions to sulfasalazine are usually due to the sulfa moiety, allergic reactions to 5-ASA can also occur.[17] Hypersensitivity reactions reported in association with ulfasalazine include skin eruptions, hemolytic anemia, neutropenia, thrombocytopenia, hepatotoxicity, pancreatitis, pericardial effusion, and interstitial nephritis.[31-33]

4.1.2 Mesalamine

Hypersensitivity to sulfa medications is a contraindication to ulfasalazine use. Mesalamine, olsalazine, and balsalazide are ulfa-free preparations of 5-ASA that can be prescribed for patients with sulfa drug allergy. Mesalamine is the 5-amino derivative of salicylic acid. Although metabolism of mesalamine to salicylic acid can occur, the formation of this metabolite is limited nd is not required for the therapeutic efficacy of this drug. Mesalamine is available in a suppository, in solution for administration as a rectal retention enema, and in oral preparations. Oral ngestion of uncoated mesalamine capsules or tablets results in xtensive absorption in the proximal gastrointestinal tract. Therefore, mesalamine is manufactured in delayed or extended release reparations to deliver 5-ASA to the mucosa of the distal gastrointestinal tract. Commercially available mesalamine products include an ethylcellulose-coated preparation (Pentasa®[1] released in he small bowel and colon)[34] and mesalamine coated with Eudragit S, an acrylic based resin (Asacol®; released in colon). The Eudragit S coated delayed-release form of mesalamine has been evaluated for maintenance of remission in pediatric IBD.[17] In children previously treated with sulfasalazine, mesalamine was well tolerated and was as efficacious as sulfasalazine in maintenance of remission.[17] In adults, the combination of oral Asacol® and rectal mesalamine suppositories was superior to Asacol® alone in patients with mild to moderate distal ulcerative colitis.[35] Mesalamine suppositories alone were effective as maintenance therapy in adults with ulcerative proctitis.[36] In a placebo-controlled trial, the mean time to relapse of rectal bleeding or diarrhea plus endoscopic inflammation was 453 days with daily mesalamine suppositories, and 158 days in the placebo group.[36] Mesalamine is generally well tolerated, but occasional adverse effects with a paradoxical worsening of symptoms can occur.[18]

4.1.3 Olsalazine

Olsalazine (Dipentum®) is the sodium salt of a salicylate, disodium 3,3-azobis (6-hydroxybenzoate), which is bioconverted to 5-ASA *in vivo*. Olsalazine 3g daily was as effective as mesalamine 3g daily for achieving endoscopic remission in adults with mild to moderate active ulcerative colitis.[37] A randomized controlled trial of pediatric patients with mild to moderate active ulcerative colitis revealed that olsalazine was well tolerated, but less effective than sulfasalazine for the treatment of acute colitis.[19] Only 39% of pediatric patients treated with olsalazine 30 mg/kg/day were in remission after 3 months of treatment, compared with 79% of patients treated with sulfasalazine 60 mg/kg/day.[19]

4.1.4 Balsalazide

Balsalazide (Colazal®) is a prodrug that contains 5-ASA and 4-aminobenzoyl-β-alanine which are joined by an azo bond. Free 5-ASA is released after metabolism by colonic bacterial azoreductases. Balsalazide 6.75 g/day produced resolution of symptoms after 12 weeks of therapy in 88% of adults, compared with 57% with mesalamine 2.4g daily.[20] Balsalazide has not been extensively evaluated in pediatric patients.

4.2 Corticosteroids

In published studies, the majority of patients with moderate colitis have required more than just 5-ASA therapy in order to achieve clinical remission. Corticosteroid therapy is usually prescribed in conjunction with 5-ASA compounds in patients with moderate to severe colitis.[38] In one study, 20 children with an average disease activity of moderate colitis were treated with corticosteroids and mesalamine 20 to 40 mg/kg/day.[39] The patients with pancolitis (two-thirds of the patients enrolled) also received prednisolone 1 to 2 mg/kg/day up to 40mg daily, and those with distal colitis were given soluble prednisolone 10mg

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daily as a retention enema. Although 17 of 20 patients were in clinical remission after 8 weeks of therapy, only eight patients achieved complete endoscopic remission.[39] Thus, clinical remission may not always correlate with endoscopic resolution of disease activity and prolonged therapy may be required before complete remission is achieved.[39] Typically, corticosteroid therapy is given for 4 to 8 weeks, then the prednisolone dose is reduced by 5 mg/day each week until corticosteroid therapy is discontinued. Others recommend gradually tapering the corticosteroid dose to an alternate day regimen.[40] Corticosteroids are effective in the treatment of active ulcerative colitis, but they do not decrease the frequency of recurrence of symptoms in patients with quiescent disease.[21]

Complications such as growth suppression, hypertension, myopathy, decreased bone mineral density, cataracts, glaucoma, and hyperglycemia have been reported with long-term corticosteroid therapy.[21] Due to the concerns about adverse effects of prolonged corticosteroid therapy on impaired linear growth, and delayed puberty in children and adolescents, corticosteroid-sparing immunomodulators (see section 4.3) have been safely and effectively used in the long-term management of pediatric ulcerative colitis.[41] However, the risks and benefits of this immunomodulatory therapy must be weighed against those of surgical therapy for ulcerative colitis.

4.3 Azathioprine and Mercaptopurine

In pediatric patients with corticosteroid-refractory or corticosteroid-dependent ulcerative colitis, or who have significant corticosteroid adverse effects, immunomodulator therapy is often prescribed. Azathioprine or mercaptopurine are the most frequently prescribed immunomodulators used as corticosteroid-sparing adjunctive therapy,[41] although there are no controlled data in children. Azathioprine is a purine analog immunosuppressive antimetabolite that is cleaved *in vivo* to mercaptopurine in the liver. Its mechanism of action is unknown. Up to 3 to 6 months of therapy is required before full clinical response is achieved.[21] Azathioprine therapy for 3 months at a dosage of 2 mg/kg/day resulted in complete response in two-thirds of children with ulcerative colitis.[42] Patients received concomitant corticosteroid therapy, and the dose of corticosteroids was reduced in all children treated with azathioprine.[42] A retrospective study of azathioprine or mercaptopurine use in pediatric ulcerative colitis also noted that corticosteroids could be discontinued in 75% of patients who were previously corticosteroid-dependent.[43] The mean time until discontinuation of corticosteroids was 11.2 months, with a range of 2 to 39 months.[43]

Careful monitoring of patients treated with azathioprine is recommended in order to detect adverse effects associated with this medication. The major adverse effects of azathioprine include bone marrow suppression, leucopenia, increased infection risk, hepatotoxicity, and pancreatitis.[43] Commercial assays exist to monitor two major metabolites of azathioprine (6-thioguanine and 6-methylmercaptopurine). 6-Thioguanine levels (230 to 450 pmol/8×10^8 erythrocytes) correlate with clinical response, while 6-methylmercaptopurine levels (>5700 pmol/8×10^8 erythrocytes) are associated with hepatotoxicity.[22] Measurement of azathioprine metabolite concentrations in serum of children with IBD reveals that many children have 6-thioguanine levels below the recommended concentration, and demonstrates that monitoring can assist in the proper administration of this medication.[22] The determination of thiopurine methyltransferase (TPMT; converts azathioprine to 6-thioguanine and 6-methylmercaptopurine) genotype for individual patients may also aid the clinician in identifying individuals at increased risk of drug-induced toxicity.[22]

4.4 Methotrexate

Methotrexate, a folic acid analog immunosuppressive antimetabolite, has been reported to reduce disease activity and corticosteroid requirements in adults with ulcerative colitis.[23] However, a randomized controlled trial of adults revealed that methotrexate was no more effective than placebo in the treatment of acute ulcerative colitis, or in the maintenance of remission.[24] Methotrexate is effective adjunctive maintenance therapy in oligoarticular juvenile rheumatoid arthritis.[25] It is possible that methotrexate may benefit subgroups of patients with extraintestinal complications such as arthritis. More studies of methotrexate are needed to determine its optimum use in pediatric ulcerative colitis.

4.5 Cyclosporine

Cyclosporine (a metabolite from the fungus *Beauveria nivea*) is an immunosuppressant cyclic polypeptide containing 11 amino acids. Cyclosporine was initially used in pediatric ulcerative colitis for fulminant disease where the need for surgery was imminent.[26] Pediatric patients who passed >5 bloody stools daily after treatment with intravenous methylprednisolone (1 to 2 mg/kg/day), and at least 10 days of bowel rest with parenteral nutrition, were treated with cyclosporine 4.9 to 9.6 mg/kg/day to achieve serum cyclosporine concentrations of 150 to 300 µg/L.[26] Eighty percent of patients responded within 2 to 9 days with resolution of rectal bleeding and decreased stool frequency to <3 bowel movements daily. Nonetheless, within 1 year, 72% of patients treated with cyclosporine underwent colectomy due to recurrence of active colitis.[26] Due to the high rate of relapse of symptoms associated

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with weaning of cyclosporine therapy and the delayed onset of action of azathioprine, the combination of cyclosporine and azathioprine therapy was used for the treatment of corticosteroid-resistant patients with fulminant colitis.[27] Cyclosporine was initially given intravenously at a dosage of 100 to 200 μg/kg/day, and then 4 to 10 mg/kg/day orally to achieve levels of 100 to 200 μg/L. Azathioprine or mercaptopurine were given concomitantly in addition to corticosteroids and 5-ASA. Cyclosporine therapy was continued for 3 to 10 months. There was a rapid clinical response in seven of eight patients, four of whom were maintained in remission with azathioprine for 2 to 5 years, and then were able to discontinue prednisone and cyclosporine.[27]

4.6 Tacrolimus

Tacrolimus is an immunosuppressant macrolide that is produced by *Streptomyces tsukubaensis*. In one trial, tacrolimus was given at a dosage of 0.1 mg/kg/dose twice daily and adjusted to achieve serum concentrations of 10 to 15 μg/L in children with corticosteroid-refractory ulcerative colitis.[28] Azathioprine or mercaptopurine was added 4 to 6 weeks after the initiation of tacrolimus therapy. An initial response occurred in 70% of patients, but only 38% were in remission after 1 year, while the remainder of the patients required colectomy.[28] Thus, as with cyclosporine therapy, tacrolimus is most effective in rapidly improving symptoms, which then allows reduction in corticosteroid therapy, improved nutrition, and psychological adaptation in preparation for colectomy that is necessary in a high percentage of these patients.

4.7 Infliximab

Infliximab is a chimeric IgG monoclonal antibody to tumor necrosis factor (TNF)-α. TNF was noted to be a cofactor in the production of inflammatory cytokines, γ-interferon and IL-2.[29] Infliximab has primarily been studied in the treatment of Crohn's disease but, recently, interest has developed in studying its potential role in the treatment of ulcerative colitis. Infliximab produced clinical, endoscopic, and histologic improvement in one preliminary study of eight adults with refractory ulcerative colitis for whom colectomy was indicated.[30] Patients also received corticosteroids, 5-ASA and mercaptopurine. All eight patients were able to taper their prednisone dose without relapse of symptoms. A preliminary study of infliximab in pediatric ulcerative colitis revealed clinical improvement within 2 days to 2 weeks after administration of infliximab 5 mg/kg by intravenous infusion.[44] Corticosteroids were discontinued in 7 of 10 children after response to infliximab therapy, but one patient required colectomy due to fulminant colitis.[44] Further studies are indicated to determine the tolerability and efficacy of infliximab therapy in the treatment of pediatric ulcerative colitis; therefore, it is currently recommended that infliximab only be used in the context of a clinical trial.

5. Nutritional Therapy

Maximizing nutritional status is extremely important in children with IBD, especially in preventing growth failure and ensuring that children achieve their genetic height potential. In children with ulcerative colitis, nutritional therapy plays an important adjunctive role in the treatment of patients who are malnourished, and in pre- and post-operative management. However, the benefit of nutrition as primary therapy for the treatment of ulcerative colitis has not been proven.[45] Enteral or parenteral nutritional supplementation, in addition to corticosteroid therapy, does not also appear to increase the remission rate or reduce the need for colectomy in ulcerative colitis.[46] Patients with severe colitis may benefit from a low fiber diet until the inflammation is controlled, while a high fiber diet is beneficial in patients with distal proctitis and constipation.

6. Fulminant Colitis

Fulminant colitis is caused by a transmural extension of inflammation to the serosa. Presenting symptoms include abdominal distention and tenderness, high fever, and hemorrhage requiring transfusions. Patients with toxic megacolon exhibit the above findings in addition to significant colonic dilatation. These conditions usually occur with extensive colitis, but may also occur with left-sided colitis. Predisposing factors for toxic megacolon include infectious colitis with cytomegalovirus or *Clostridium difficile*, electrolyte abnormalities such as hypokalemia, medications such as anticholinergics or opiates, or invasive diagnostic procedures such as colonoscopy or barium enema.

When the diagnosis of toxic megacolon is made, surgical consultation should be obtained. Medical therapy includes the administration of intravenous crystalloid and colloid for support of fluid and electrolytes, blood transfusions, fresh frozen plasma if clotting abnormalities are present, intravenous corticosteroids such as prednisolone, methylprednisolone, or hydrocortisone, and intravenous antibiotics.[47] Continuous intravenous cyclosporine (2 to 4 mg/kg/day) has been used in adults as an alternative option to emergent colectomy,[48] but no data are available in children. Enteral intake should be discontinued and nasogastric suction should be utilized to reduce distention. Additional methods that can be used to reduce distention include insertion of a rectal tube, rolling the patient from side to side, or using prone knee to chest positioning.[49] Abdominal radiographs should be obtained at

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least daily to assess for signs of perforation, which is an immediate indication of colectomy. Colectomy should also be considered if there is no improvement within 72 hours.

7. Cancer in Ulcerative Colitis

In longitudinal studies of patients with ulcerative colitis, the earliest cases of colon cancer occurred at least 10 years after the time of diagnosis.[50] Therefore, it is recommended that colonoscopy for colon cancer screening begin 8 years after diagnosis, then every 1 to 2 years thereafter.[51] Onset of ulcerative colitis before the age of 15 years was associated with a higher incidence of colon cancer (40%) than in those whose onset of symptoms occurred after 15 years of age.[52]

One potential extraintestinal manifestation of ulcerative colitis is sclerosing cholangitis. The estimated frequency of primary sclerosing cholangitis in patients with ulcerative colitis is 2 to 5%, while the estimated frequency of ulcerative colitis in patients with primary sclerosing cholangitis is 67%.[53] Patients with both ulcerative colitis and primary sclerosing cholangitis are at higher risk of colonic dysplasia and cancer, which approaches 50% after 25 years of disease. However, the severity of ulcerative colitis does not correlate with the severity of primary sclerosing cholangitis, and vice versa.[53] Ursodeoxycholic acid is prescribed to patients with primary sclerosing cholangitis. It was observed in a cross-sectional study, that patients with ulcerative colitis who received ursodeoxycholic acid had a lower incidence of colonic dysplasia than patients who did not receive this medication, suggesting that ursodeoxycholic acid may have a chemoprotective effect against the development of colon cancer in ulcerative colitis.[54]

The extent and duration of disease influence the risk of developing colon cancer in patients with ulcerative colitis. For patients with pancolitis, the risk of colon cancer was observed to be 30% after 35 years of the disease.[55] This increased risk of colon cancer is one factor to be considered when contemplating colectomy for these patients.

8. Surgical Management

Although colectomy is curative for ulcerative colitis, the majority of pediatric patients can be adequately managed with medical therapy and do not require surgery during childhood or adolescence. In a study of 171 pediatric patients with ulcerative colitis, the need for colectomy at 1 year following diagnosis was 1% among those with mild disease versus 8% with moderate/severe disease; at 5 years, the risk was 9% versus 26% for the two groups.[16] The indications for colectomy are intractable disease in 64% of patients, growth failure in 14%, toxic megacolon in 6%, uncontrolled hemorrhage in 4%, perforation in 3%, and cancer prophylaxis in 2%.[56]

The primary benefit of colectomy for ulcerative colitis is the control of symptoms without the need for maintenance medications. Another benefit of surgery for ulcerative colitis is improved growth postoperatively in prepubertal patients.[57] In one study, the linear growth velocity increased from 3.8 to 7.3 cm/year in 11 of 18 children after colectomy.[57]

Major complications after surgery for pediatric ulcerative colitis (wound infection, bowel obstruction, prolonged fever, pouchitis, pouch fistula) are common (53%),[58] but occur primarily in patients with immunosuppression. Symptoms reported after colectomy include increased stool frequency, fecal incontinence with inability to distinguish flatus from stool, pouchitis, and stricture or fistula formation at the ileo-anal anastomosis site in patients subsequently diagnosed with Crohn's disease.[59] Consideration of these potential postoperative symptoms versus the symptoms of active colitis influences the decision about whether to defer or recommend colectomy for pediatric patients with ulcerative colitis. Nonetheless, with a mean follow-up of 5.8 ± 3.3 years, 90% of patients report satisfaction with the functional outcome of ileoanal anastomosis surgery after colectomy for ulcerative colitis.[59] Despite the potential benefits of surgery, the reported frequency of colectomy in children has decreased, probably owing to improved medical therapy for ulcerative colitis.[60]

9. Conclusions

Diagnosis of ulcerative colitis in children and adolescents requires a high index of suspicion and performance of sufficient diagnostic testing in patients with suggestive signs and symptoms.

The treatment of pediatric ulcerative colitis differs from that in adults in that maintenance of growth and pubertal development is an important goal in disease management in children. Ulcerative colitis can be managed with 5-ASA in many patients, but corticosteroids are often required for the treatment of moderate to severe acute colitis. Immunomodulator therapy with azathioprine or mercaptopurine is increasingly used for long-term corticosteroid-sparing maintenance therapy in pediatric ulcerative colitis for patients with frequent relapsing symptoms. These drugs are not effective in acute colitis. However, other immunomodulators, such as cyclosporine or tacrolimus, are effective in acute colitis, but are associated with a high rate of disease relapse upon withdrawal of these medications. Monoclonal antibody therapy with infliximab may also be beneficial in the management of ulcera-

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tive colitis, but further studies are needed to determine its tolerability and efficacy in children.

The need for, or timing of, colectomy in children is determined by weighing the response to medical therapy, risks of growth impairment, and ultimate cancer risk against the prospects of surgical cure, along with potential adverse effects after surgery.

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Topical cyclosporin treatment of keratoconjunctivitis sicca in secondary Sjögren's syndrome

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Abstract. Topical cyclosporin 2% in olive oil was investigated for its possible immunoregulatory role on the dry eye state in patients with secondary Sjögren's syndrome. The study was a randomized, double-masked, placebo-controlled trial. Thirty eyes of 15 patients were randomized to undergo treatment with topical cyclosporin in olive oil and 30 eyes of the other 15 patients received a placebo, which was the sterile olive oil used as a vehicle for the cyclosporin. The effect of the 2-month long treatment with either medication on the status of the dry eye state was measured by Schirmer-I test, tear film break-up time and rose bengal staining. There was a significant increase in the break-up time and a significant decrease in rose bengal staining score between the cyclosporin and control groups at the end of the 2-month study period ($p < 0.01$). Schirmer-I test remained unaffected ($p > 0.05$). These results probably indicate that topical cyclosporin modulates the goblet cell function in secondary Sjögren's associated keratoconjunctivitis sicca and through this mucus enhancing action or some other mechanism not yet known, helps to maintain the structural integrity of the epithelium.

Key words: cyclosporin A - controlled clinical trial - keratoconjunctivitis sicca - Sjögren's syndrome.

Keratoconjunctivitis sicca (KCS) is a symptom complex of all the conditions which cause abnormalities of tear film flow and/or stability. The instability of the tear film results from the deficiency of any of the tear components (mucin, aqueous and lipid) (Bron 1985; Manthorpe et al. 1986; Prause et al. 1986; Whitcher 1987).

Within a population of patients with dry eyes, three subgroups exist: idiopathic KCS (no xerostomia), primary Sjögren's syndrome (xerostomia present) and secondary Sjögren's syndrome (xerostomia and/or connective tissue disorder present). Idiopathic KCS without any associated abnormality is probably the most common of these three conditions (Holly 1986).

The most commonly used treatment for dry eye is still the frequent instillation of an artificial tear substitute or replacement. Most commercially available artificial tear preparations have short retention times and therefore require frequent instillation. Furthermore, some of the preservatives such as benzalkonium chloride can be counterproductive and decrease the stability of the tear film, causing a superimposed keratitis medicamentosa. In severely affected cases punctal occlusion, slow-release devices and intracanalicular collagen inserts can be valuable, though these treatment modalities are not without problems (Lemp 1973, 1987; Snibson et al. 1992).

Pathogenesis of KCS encompasses alterations in both the humoral and cellular aspects of the immune system (Farris et al. 1991; Forstot et al. 1981). In this context, topical cyclosporin was investigated for its probable immunomodulatory role in dry eye cases with secondary Sjögren's syndrome.

Materials and Methods

The project was designed as a randomized, double-masked, placebo controlled study to investigate the effect of topical cyclosporin 2% in olive oil on keratoconjunctivitis sicca in secondary Sjögren's syndrome. After obtaining permission

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from the responsible committee on human experimentation, the nature of the drug to be used was fully explained to the patients involved in the study and a written permission was requested from each. Topical cyclosporin was prepared under sterile conditions using commercially available cyclosporin 100 mg/ml solution diluted in olive oil.

The patient population consisted of 30 patients with secondary Sjögren's syndrome. The patients were randomized to treatment with study medication of either cyclosporin 2% olive oil solution or placebo (olive oil vehicle without medication). Thirty eyes of 15 randomly selected patients were prescribed topical cyclosporin 2% in olive oil 4 times daily. The placebo group consisted of 30 eyes of the other 15 patients started on olive oil used as the carrier vehicle of cyclosporin.

Carboxypolymethylene gel tear was also administered 4 times daily to overcome the initial discomfort experienced with cyclosporin or olive oil solution and prevent the discontinuance of the drug. The patients had in fact been using gel tears as part of the treatment for KCS and this medication was not stopped when they enrolled in this study protocol. The use of other concomitant oral or topical medications that could affect tear production or interfere with the metabolism of cyclosporin was prohibited throughout the trial.

The effect of topical cyclosporin in olive oil and olive oil vehicle per se on the status of the dry eye state was measured by Schirmer-I test, tear film break-up time and rose bengal staining. The tests were done at the beginning and at the end of the 2-month study period. The results of the tests for the 2 eyes of 1 patient were averaged to give a single value. The mean values for each group was thus calculated by averaging 15 measurements.

In Schirmer-I test, a standardized test strip was placed at the outer one-third of the lower lid. No topical anesthesia was used. The patients were informed that they could close but not squeeze their eyes. After the test, the length of the wetted strip was measured using a gauge. Wetting less than 5 mm indicates impaired secretion and values less than 10 mm are suspicious (Clinch et al. 1983; Taylor & Louis 1980; van Bijsterveld 1969).

Tear film break-up time test was done by applying one drop of 1% fluorescein solution to the lower fornix. The patient was told to blink once to enable the even spread of fluorescein over the ocular surface. The time interval between the last blink and the first appearance of a dry spot on the precorneal tear film was recorded. This measurement was repeated 3 times and the results were averaged. A break-up time of 10 sec or less is generally considered to be abnormal though in some normal subjects dry spots may appear in less than 10 sec (Holly 1987; Norn 1969; Vanley et al. 1977).

Staining with rose bengal was performed on non-anesthetized eyes. After instillation of 1% rose bengal into lower fornix and waiting for 5 to 10 min, the staining score was recorded. The lateral and medial exposed areas of conjunctiva and the cornea were examined separately under red-free light. The number of stained spots in each of the 3 areas was determined and scored from 0 to 3, resulting in a total score from 0 to 9 in each eye. A score above 3 or 3.5 in an eye indicates a pathological condition (Woldoff & Haddad 1973; Norn 1970).

All the patients were monitored to detect any systemic side effects of cyclosporin therapy. Blood urea nitrogen, creatinine levels, liver function tests, erythrocyte sedimentation rate, uric acid levels and complete blood count were periodically checked. Whole blood levels of cyclosporin were measured using the high-performance liquid chromatography (HPLC) method on a biweekly basis.

Results

The patient population consisted of 27 women and 3 men. The mean age was 52.1 ± 1.1 years. Of the 30 patients, 22 had rheumatoid arthritis, 2 had systemic lupus erythematosus, 2 had scleroderma, 2 had Crest syndrome and 2 had polymyositis.

The mean values of Schirmer-I test, tear film break-up time and rose bengal staining scores for the cyclosporin and control groups at the beginning of the study are shown in Table 1. The patients were effectively randomized into the cyclosporin and control groups and there is no meaningful difference in the initial test results between the two groups. The mean values of Schirmer-I test, tear film break-up time and rose bengal staining at the end of the study period of 2 months are given in Table 2. There was a statistically significant increase in the break-up time and a significant decrease in rose bengal staining score in the cyclosporin group as compared to the placebo (olive oil) group (Student's t-test, $p < 0.01$). Schirmer-I test

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Table 1.

Comparison of mean values of Schirmer-I test, break-up time and rose bengal scores of the cyclosporin and control groups at the initial examination using the Student's t-test.

Test	Cyclosporin	Olive oil	Significance
Schirmer-I (mm)	5.63 ± 0.82	5.42 ± 0.62	$p > 0.05$
Break-up time (sec)	6.15 ± 0.88	5.80 ± 0.72	$p > 0.05$
Rose bengal score	5.10 ± 0.53	5.35 ± 0.65	$p > 0.05$

Table 2.

Comparison of mean values of Schirmer-I test, break-up time and rose bengal scores of the cyclosporin and control groups at the end of the study period of 2 months using the Student's t-test.

Test	Cyclosporin	Olive oil	Significance
Schirmer-I (mm)	5.85 ± 0.92	5.50 ± 0.72	$p > 0.05$
Break-up time (sec)	8.52 ± 1.01	5.70 ± 0.80	$p < 0.01$
Rose bengal score	3.38 ± 0.39	5.20 ± 0.62	$p < 0.01$

esults did not show any meaningful difference between the two groups ($p > 0.05$).

Mild discomfort was observed in three of the eyes undergoing cyclosporin treatment and in two of the eyes receiving olive oil solution. This effect was transient and none of the patients discontinued the treatment because of it. No other ocular side effects related to the use of topical cyclosporin or olive oil was noted. Blood cyclosporin levels were below 50 ng/ml in all the patients with the HPLC method. Complete blood count, sedimentation rate, blood pressure, blood urea nitrogen, creatinine levels, liver function tests and uric acid levels were within normal limits.

Discussion

opical cyclosporin

Cyclosporin A, a cyclic peptide produced by the ungi Tolypocladium Inflatum Gams, is a powerful mmunomodulator. The most important cellular ffect of cyclosporin is by forming a pentameric omplex with cyclophilin, calcineurins and calmodulin which inhibits intracellular protein synthesis nd consequently the interleukin 2 gene activation n T-cells (Wiskott 1993). The result is the inhibition of proliferation, function and interaction of ne various immunocompetent cells. B-cells, mast cells and several cytokines are inhibited by cyclosporin (Holland et al. 1993).

The mechanism of action of topical cyclosporin has not been fully understood. Cyclosporin is a hydrophobic peptide and it easily penetrates the intact hydrophobic corneal epithelium to accumulate on the stroma (Diaz-Llopis & Menezo 1989). Topical cyclosporin has been used in the management of a variety of anterior segment inflammatory disorders that have failed conventional management. High-risk keratoplasty (Belin et al. 1989; Gündüz & Özdemir 1993; Holland et al. 1993), ligneous conjunctivitis (Holland et al. 1989, 1993) atopic and vernal conjunctivitis (Ben Ezra et al. 1986, 1993), ulcerative keratitis (Holland et al. 1993) and Mooren's ulcer (Holland et al. 1993; Zhao J & Jin X 1993) have been managed successfully with partial or total control of inflammation using topical cyclosporin. Ben Ezra et al. (1993) reported that the only indication for topical cyclosporin was vernal conjunctivitis and that the therapeutic response was difficult to predict in the other indications.

Several preparations of topical cyclosporin solution have been made. The standard 100 mg/ml oral solution can be diluted to a 2% topical solution using sterile olive or corn oil (Belin et al. 1989; Holland et al. 1993). Alternatively, the 50 mg/ml intravenous solution of cyclosporin can be diluted to a 1% solution using artificical tears (Holland et al.

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1993) or diluted to a 0.5% solution using 0.9% sodium chloride solution (Zhao J & Jin X 1993). Cyclosporin 1% ophthalmic corn oil ointment has also been used (Laibovitz et al. 1993) for topical treatment.

The only drawback to the use of topical cyclosporin is local irritation. The concomitant use of artificial tears help alleviate the irritative symptoms. Transient epitheliopathy has been reported to develop after topical cyclosporin usage (Belin et al. 1989). Scanning electron microscopic study of rabbit corneas treated with topical cyclosporin revealed focal areas of epithelial cell loss (Versura et al. 1989). These changes were present in both the cyclosporin treated rabbits and in those receiving olive oil only, suggesting that oil as a vehicle might be responsible for the epithelial damage. However, corneal healing was found to be unaffected with topical cyclosporin in the animal model (Kossendrup et al. 1986). In another study, irritative reactions were found to decrease when corn oil was substituted for olive oil (Kaswan et al. 1989).

Treatment of KCS in secondary Sjögren's syndrome using topical cyclosporin

Keratoconjunctivitis sicca is generally believed to result from lymphocytic infiltration of the lacrimal gland with destruction of the acinar and ductal structures, resulting in a deficiency of the aqueous layer of the precorneal tear film. The principal cell of this inflammatory infiltrate is the T-helper/inducer cell. KCS patients also have fewer goblet cells in their conjunctiva than normal which results in the formation of an abnormal mucus layer (Greaves et al. 1991; Tseng et al. 1984). Hyperactivity of both the humoral and cellular aspects of the lymphoid system is seen in KCS and patients with KCS frequently have circulating antibodies, immune complex disease and lymphocytic proliferation (Farris et al. 1991; Forstot et al. 1981).

Since the principal inflammatory cell in KCS is the T-helper/inducer lymphocyte and cyclosporin has an inhibitory effect on these cells, topical cyclosporin has been tried in the treatment of patients with secondary Sjögren's syndrome. There have been few reports to date on the use of topical cyclosporin in this indication (Kaswan et al. 1989; Power et al. 1993; Laibovitz et al. 1993). Power et al. demonstrated that topical cyclosporin may have a local immunosuppressive effect on the conjunctiva in patients with Sjögren's associated keratoconjunctivitis sicca. Epithelium and substantia propria in the Sjögren patients before treatment showed significantly more helper/inducer T (CD4+) cells than age and sex-matched controls. Following treatment with topical cyclosporin, there was a significant reduction in the number of helper/inducer T (CD4+) cells in both the conjunctival epithelium and substantia propria. This effect of topical cyclosporin is probably because of its local immunosuppressive action and not because of systemic absorption, since topical application of cyclosporin drops does not produce detectable levels in the plasma. Laibovitz et al. found that rose bengal results and subjective discomfort parameters favored cyclosporin over olive oil in a comparative trial conducted with keratoconjunctivitis sicca patients.

Break-up time and rose bengal staining scores differed significantly between the cyclosporin and control groups at the end of the 2-month study period in our trial. There was, on the other hand, no statistically significant difference in the results of the Schirmer-I test. Significant improvement in break-up time and no change in Schirmer-I test show that topical cyclosporin modulates goblet cell function more than the lacrimal gland function. This means topical cyclosporin is effective on the ocular surface where goblet cells are abundant, but its effect on the lacrimal secretory/drainage system is rather remote. Rose bengal stains epithelial cells with damaged cell membranes, reflecting directly the degree of mucosal surface destruction. Therefore topical cyclosporin also contributes to the structural integrity of the conjunctival and corneal epithelium, either by way of its mucus stabilizing properties or through some other immunomodulating mechanism not yet elucidated. A larger study might better define the role of topical cyclosporin in dry eye states.

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Polyethylene glycol ester

GENERAL PROPERTY

Composition	Appearance	Acid Value (max.)	Saponification Value	Melting Point	Cloud Point (1% aq. soln.) (¡É)	Specific Gravity (a) at 25¡É (b) at 60¡É	HLB
PEG 200 Monolaurate	Liquid	5	132~142	< 5	15	0.9833(a)	9.3
PEG 200 Dilaurate	Liquid	10	176~186	< 9	< 5	0.9520(a)	5.9
PEG 300 Monolaurate	Liquid	5	104~114	< 8	46	1.0100(a)	11.4
PEG 300 Dilaurate	Liquid	10	148~158	< 13	< 5	0.9703(a)	7.9
PEG 400 Monolaurate	Liquid	5	86~96	12	68	1.0242(a)	13.0
PEG 400 Dilaurate	Liquid	10	127~137	18	29	0.9884(a)	9.7
PEG 600 Monolaurate	Liquid	5	64~74	23	68	1.0505(a)	14.6
PEG 600 Dilaurate	Soft solid	10	102~112	24	30	0.9820(b)	11.7
PEG 1000 Monolaurate	Soft solid	5	41~51	40	88	1.035(b)	16.6
PEG 1000 Dilaurate	Soft solid	10	68~78	38	60	1.015(b)	14.2
PEG 1540 Monolaurate	Wax	5	26~36	46	> 100	1.060(b)	17.5
PEG 1540 Dilaurate	Wax	10	48~56	42	73	1.040(b)	15.8
PEG 4000 Monolaurate	Wax	5	9~18	55	> 100	1.075(b)	19.0
PEG 4000 Dilaurate	Wax	5	20~30	52	83	1.065(b)	18.1
PEG 6000 Monolaurate	Wax	5	7~13	61	> 100		19.3
PEG 6000 Dilaurate	Wax	9	12~20	57	96	1.077(b)	18.7
PEG 200 Monostearate	Solid	5	120~129	31	< 5	0.9360	8.1
PEG 200 Distearate	Solid	10	153~162	34	< 5	0.9060	4.8
PEG 300							

PTO-000647

Monostearate	Solid	5	97~105	28	< 5	0.9060	10.3
PEG 300 Distearate	Solid	10	130~139	32	< 5		6.9
PEG 400 Monostearate	Solid	5	83~92	32	< 5	0.9780	11.7
PEG 400 Distearate	Solid	10	115~124	36	< 5	0.9390	8.5
PEG 600 Monostearate	Solid	5	61~70	37	55	1.0000	13.5
PEG 600 Distearate	Solid	10	93~102	39	< 5	0.9670	10.7
PEG 1000 Monostearate	Wax	5	40~48	41	91	1.030	15.7
PEG 1000 Distearate	Wax	10	65~74	40	51	1.005	13.3
PEG 1540 Monostearate	Wax	5	27~36	47	> 100	1.050	16.9
PEG 1540 Distearate	Wax	10	47~58	45	78	1.015	14.6
PEG 4000 Monostearate	Wax	5	10~18	56	> 100	1.075	18.7
PEG 4000 Distearate	Wax	5	19~27	5	91	1.060	17.6
PEG 6000 Monostearate	Wax	5	7~13	61	> 100	1.080	19.1
PEG 6000 Distearate	Wax	9	14~20	55	83	1.075	18.4
PEG 200 Monooleate	Liquid	5	115~124	< -15	< 5	0.9742	8.2
PEG 200 Dioleate	Liquid	10	148~158	< -15	< 5	0.9405	5.0
PEG 300 Monooleate	Liquid	5	94~102	< -5	< 5	0.998	10.2
PEG 300 Dioleate	Liquid	10	128~137	< -5	< 5	0.9609	6.9
PEG 400 Monooleate	Liquid	5	80~89	< 10	65	1.0135	11.6
PEG 400 Dioleate	Liquid	10	113~122	< 7	< 5	0.977	8.3
PEG 600 Monooleate	Liquid	5	60~69	23	80	1.0381	13.6
PEG 600 Dioleate	Liquid	10	92~102	19	10	1.0038	10.6
PEG 1000 Monooleate	Soft solid	5	40~49	39	98	1.035	15.9
PEG 1000 Dioleate	Soft solid	10	64~74	37	47	1.005	13.2
PEG 1540 Monooleate	Wax	5	28~37	45	> 100	1.050	17.0
PEG 1540							

PTO-000648

Dioleate	Wax	10	45~55	44	82	1.025	14.9
PEG 4000 Monooleate	Wax	5	10~18	55	> 100	1.075	18.7
PEG 4000 Dioleate	Wax	5	19~27	49	88	1.060	17.7
PEG 6000 Monooleate	Wax	5	7~13	59	> 100	1.085	19.1
PEG 6000 Dioleate	Wax	9	13~21	56	87	1.070	18.4

SOLUBILITY

Composition	Water	Propylene Glycol	Isopropyl Alcohol	Isopropyl Myristate	Mineral Oil	
					55 Vis	330 Vis
PEG 200 Monolaurate	D	I	S	S	PS	I
PEG 200 Dilaurate	D	I	S	S	S	S
PEG 300 Monolaurate	D	I	S	PS	I	I
PEG 300 Dilaurate	D	I	S	S	S	I
PEG 400 Monolaurate	S	I	S	PS	I	I
PEG 400 Dilaurate	D	I	S	S	I	I
PEG 600 Monolaurate	S	I	S	I	I	I
PEG 600 Dilaurate	D	I	S	S	I	I
PEG 1000 Monolaurate	S	SH	S	I	I	I
PEG 1000 Dilaurate	S	PS	S	S	I	I
PEG 1540 Monolaurate	S	SH	SH	I	I	I
PEG 1540 Dilaurate	S	SH	SH	PS	I	I
PEG 4000 Monolaurate	S	SH	SH	I	I	I
PEG 4000 Dilaurate	S	SH	SH	I	I	I
PEG 6000 Monolaurate	S	SH	SH	I	I	I
PEG 6000 Dilaurate	S	SH	SH	I	I	I
PEG 200	DH	I	S	S	I	I

Monostearate						
PEG 200 Distearate	DH	I	S	S	S	S
PEG 300 Monostearate	DH	I	S	S	I	I
PEG 300 Distearate	DH	I	S	S	S	S
PEG 400 Monostearate	DH	I	S	S	I	I
PEG 400 Distearate	DH	I	S	S	S	S
PEG 600 Monostearate	S	PS	S	PS	I	I
PEG 600 Distearate	DH	I	S	S	I	I
PEG 1000 Monostearate	S	S	S	I	I	I
PEG 1000 Distearate	S	S	S	S	I	I
PEG 1540 Monostearate	S	S	S	I	I	I
PEG 1540 Distearate	S	S	S	PS	I	I
PEG 4000 Monostearate	S	S	S	I	I	I
PEG 4000 Distearate	S	S	S	I	I	I
PEG 6000 Monostearate	S	S	S	I	I	I
PEG 6000 Distearate	PS	S	S	I	I	I
PEG 200 Monooleate	D	I	S	S	S-1%	I
PEG 200 Dioleate	D	I	S	S	S	S
PEG 300 Monooleate	D	I	S	PS	I	I
PEG 300 Dioleate	D	I	S	S	S	S
PEG 400 Monooleate	D	I	S	PS	I	I
PEG 400 Dioleate	D	I	S	S	S	I
PEG 600 Monooleate	S	I	S	I	I	I
PEG 600 Dioleate	D	I	S	S	I	I
PEG 1000 Monooleate	S	SH	S	I	I	I
PEG 1000						

PTO-000650

Dioleate	S	I	S	PS	I	I
PEG 1540 Monooleate	S	S	SH	I	I	I
PEG 1540 Dioleate	S	S	SH	I	I	I
PEG 4000 Monooleate	S	S	SH	I	I	I
PEG 4000 Dioleate	S	S	SH	I	I	I
PEG 6000 Monooleate	S	S	SH	I	I	I
PEG 6000 Dioleate	S	S	SH	I	I	I

S : Soluble, I : Insoluble, D : Dispersible, PS : Partial soluble, DH : Dispersible(hot), SH : Soluble(hot)

APPLICATION

- Makeup
  Pigment wetting and dispersing
  PEG 200 Monooleate, PEG 200 Dioleate and PEG 400 Monooleate, PEG 400 Dioleate

- Bath oils, Bath lotions and After Bath product
  Emulsifiers and lubricants
  PEG 400 Monooleate, PEG 400 Dioleate and PEG 600 Monooleate, PEG 600 Dioleate

- Ointments
  Viscosity builders
  PEG 1540 Monostearate and PEG 4000 Monostearate

- Lotions, Shampoos and Cream rinses
  Viscosity builders
  PEG 1540 Distearate and PEG 6000 Distearate

- Creams and Lotions
  Primary and auxiliary emulsifiers
  PEG 200 and PEG 1540 esters

  Emollients
  PEG 200 ~ 600 Monolaurate, Dilaurate, Monooleate, Dioleate

- Hair Care Products
  Opacifiers
  PEG 200 Monostearate, PEG 200 Distearate, PEG 400 Monostearate and PEG 400 Distearate

  Conditioners
  PEG Stearates

PTO-000651

▫ Clear Bath Oils	Spreading agents PEG 200 Dilaurate and PEG 400 Dilaurate
▫ Suppositories	Melting point control PEG 400 Dilaurate and PEG 600 Dilaurate

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CP | HLB | POE alkyl (and alkyl aryl) ether | Sorbitan fatty acid ester | POE Sorbitan fatty acid ester | PEG | Surface tension etc.

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Cyclosporin A applied topically to the recipient eye inhibits corneal graft rejection

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SUMMARY

Corneal graft survival in rabbits was significantly ($P < 0{\cdot}001$) prolonged by topical treatment to the recipient eye with cyclosporin A 1% in arachis oil applied five times daily for 4 weeks. No graft was rejected whilst treatment was maintained but all grafts subsequently underwent rejection by the 64th postoperative day. All animals in a simultaneous control group in this fully masked study developed allograft reactions by the 23rd day. No local or systemic side-effects attributable to cyclosporin A were observed. It is believed that this is the first report of inhibition of an allograft reaction by cyclosporin A applied topically.

INTRODUCTION

Cyclosporin A (CyA) has been shown to act as a potent immunosuppressive agent in inhibiting allograft reactions in man and a variety of experimental animals, but its use in man has been associated with the development of lymphomas and nephrotoxicity (Editorial, *Lancet*, 1979; Calne *et al.*, 1978). Serious side-effects of this nature discourage its systemic use in man for non-essential organ transplants such as corneal grafting, especially in persons having another sighted eye. Corneal allograft reactions in rabbits have been shown to be either delayed or prevented by the systemic administration of CyA, but these experiments relied on skin transplantation from the same donor to provide a second stimulus to systemic sensitization, thereby ensuring prompt rejection of the graft (Coster *et al.*, 1979). Topical administration of CyA to the recipient eye using that rabbit model, in which sensitization is substantially the result of the second-set skin transplant, did not influence the onset of rejection (Shepherd *et al.*, 1980).

Nevertheless, peripheral immunological functions in the ocular tissues (Fig. 1) make it possible that topical administration of CyA to the eye receiving a corneal transplant might interfere with the early process of sensitization sufficiently to inhibit subsequent corneal graft rejection. If this proved to be the case, topical administration of CyA would require only small quantities of the compound to be administered to one eye, thereby greatly reducing the risk of adverse side-effects, and might well confine this risk to the ocular tissues, which are accessible for serial microscopic inspection *in vivo*.

In order to investigate these possibilities we have developed a new rabbit model in which sensitization and prompt rejection of the corneal allograft results purely from the corneal transplantation. We report here the results of topical administration of CyA in this model.

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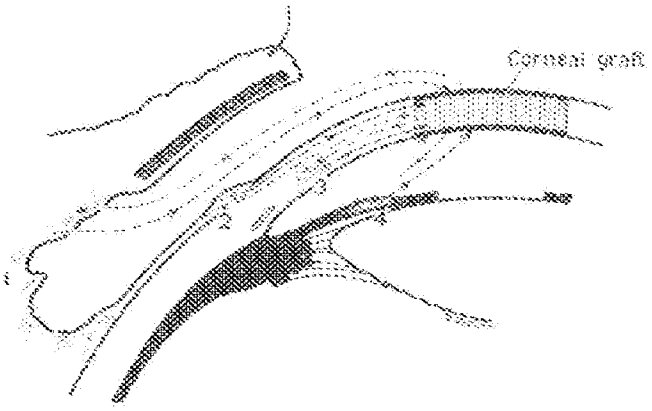

Fig. 1. Possible routes of local immune reactions to corneal transplants (Jones, 1973). Lymphoid cells in: (1) conjunctiva, (2) limbus, (3) cornea, (4) uveal tract.

MATERIALS AND METHODS

Outbred strains of Dutch and New Zealand white rabbits weighing 1–2·5 kg were used in pairs to perform corneal allografts or singly for autografts. Prior to operation all animals were fully anaesthetized with intravenous pentobarbitone together with amethocaine drops to the eye, and received intravenous heparin, 2,500 units/kg, and topical mydriatics. Corneal grafts 6 mm in diameter were exchanged between Dutch and New Zealand white rabbits. The graft was positioned eccentrically with its periphery 1–2 mm from the edge of the cornea in order to ensure maximum vascularization of the graft. The grafts were secured with a 10/0 monofilament nylon continuous suture which was left in place for 14 days. Iris adhesions were not divided at the time of operation, thereby providing an additional stimulus for vascularization. Only animals with uncomplicated operations in which the corneal grafts cleared promptly after operation were included in the study. Autografts were performed in an identical manner except that the corneal disc was sutured back into the same animal.

All animals received topical mydriatic drops and antibiotic ointment once daily for 14 days following operation and were examined each day using the slit-lamp. The onset of corneal graft rejection in the allograft groups was diagnosed by the appearance of epithelial or endothelial rejection lines on the graft which, in the latter case, was associated with the development of corneal stromal oedema or infiltrate in the previously clear graft. This diagnosis was confirmed in each case by observing typical progression. Once rejection was established the animal was killed and the eye removed for histological examination. The graft survival time was defined in each case as the time which elapsed from the date of the operation until the first clinical sign of rejection appeared in the graft.

Two groups of animals also received either topically applied CyA 1% in arachis oil or arachis oil alone administered on a fully masked basis to the recipient eye. The drops were given five times daily, with the first dose given at the conclusion of the operation, and were continued for 28 days unless rejection was diagnosed sooner.

The animals were weighed regularly and examined clinically for signs of local or systemic toxicity.

The results have been analysed using Student's *t*-test and standard log rank analysis.

RESULTS

The rabbits were divided into four groups according to the type of graft performed and the treatment given during the postoperative period.

Group I—autografts (seven rabbits)
All the animals on which autografts were performed maintained clear grafts for periods of at least 3 months and showed none of the clinical features of rejection found in the other three groups (Fig. 2). During the first 14 days new blood vessels proliferated around and into the graft but these regressed following removal of sutures, remaining visible only as 'ghost' vessels after the 3rd or 4th week.

Group II—allografts (12 rabbits)
All animals in this group showed clinical signs of graft rejection which was subsequently confirmed by histological examination of the cornea. The first day on which any of the animals showed any signs of rejection was day 14 postoperatively and by day 34 100% of the grafts had developed signs of an allograft reaction (Fig. 2). The mean survival time for allografts in this group was 21·7 ± 5·6 days.

Group III—allografts treated with topical arachis oil (10 rabbits)
The results were essentially similar to those in group II. The development of the allograft reaction occurred between days 8 and 23 with a mean survival time of 19·2 ± 4·2 days (Fig. 2). When compared to group II this difference is not significant ($P = 0·267$).

A number of animals in this group, as in group IV, developed a variable amount of hair loss around the treated eye. This appeared to be related to incidental application of drops to the surrounding skin and subsequent scratching by the rabbit.

Group IV—allografts treated with topical cyclosporin A in arachis oil (10 rabbits)
The corneal allografts in those animals which received CyA 1% in arachis oil applied topically to the recipient eye showed prolonged survival (Fig. 2). The clinical picture in the early stages was similar to that of the autografted group, with vascularization of the graft occurring during the first 14 days and subsequent regression of active vessels during the ensuing period while treatment with CyA was maintained. Thereafter, following 1–2 days of vessel reactivation, all grafts eventually developed characteristic epithelial or endothelial rejection lines between days 31 and 64 with a mean survival time of 44·5 ± 10·1 days. When compared with groups II and III these results gave a P value of $< 0·001$ in each case.

As in group III some animals developed fur loss in the region of the operated eye but the incidence of this finding was similar in the two groups and in all cases when the treatment was stopped the hair started to regrow promptly. No local or systemic side-effects that could be directly attributable to CyA were noted.

Histopathology
Histological examination by light microscopy of the grafted eyes in groups II, III and IV showed

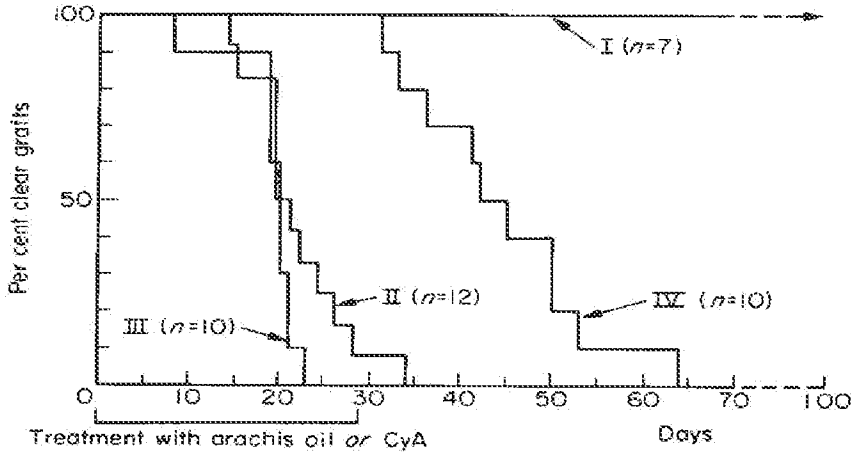

Fig. 2. Survival of rabbit corneal autografts (I) and allografts treated with topical mydriatics and antibiotics (II), with arachis oil (III) and with cyclosporin A (IV).

changes consistent with an allograft reaction similar to those previously described elsewhere (Silverstein & Khoudadoust, 1963).

DISCUSSION

These results demonstrate that CyA can inhibit corneal transplant rejection when applied topically to grafted eyes of rabbits in which it is the transplanted corneal antigens alone that have initiated sensitization. They also confirm previous reports (Borel *et al.*, 1977; White *et al.*, 1979; Markwick *et al.*, 1979) that CyA acts at an early stage in the allograft rejection process. In a different experiment, where the animals were sensitized by corneal transfer and also by a second-set skin transfer, topical application of CyA to the grafted cornea failed to prevent subsequent rejection (Shepherd *et al.*, 1980). If the drug were acting on the efferent limb of the rejection process, it would be expected to prolong allograft survival in both models.

A number of local and systemic mechanisms through which corneal allografts in man and experimental animals may be mediated have been proposed (Jones, 1973), and their relative importance may differ according to the clinical circumstances and experimental model. Systemic sensitization can occur in man following previous corneal grafting or other allogeneic transfers (Stark *et al.*, 1978) and in experimental xenografts in animals (Polack & Gonzales, 1968), but it is also possible that local factors are equally or more important in first-time corneal allografts (Polack 1966). In our model the clinical and histological features show that the bulk of the reaction occurs in the superficial layers of the cornea and surrounding limbus where it would be expected that a topically applied drug would have most access.

With the regimen of topical administration used, we did not demonstrate a prolonged effect in a proportion of animals such as has been observed with other modes of dosage in other grafting models (Coster *et al.*, 1979; Calne *et al.*, 1978; Green & Allison, 1978). Further experiments are in progress to establish whether this difference is related to the degree of immunological challenge presented in this model, to the distribution of the drug when given by various schedules or to differing sites of action when given by various routes of administration.

Corneal graft rejection still remains the main limitation to the application of corneal grafting, and is a leading cause of failure of corneal grafts (Jones, 1973). A safe method of ocular immunosuppression that is more effective than the current very prolonged topical administration of corticosteroids could thus provide a major advance in the treatment of blindness from corneal disease. This is especially the case in those parts of the world where lack of skilled postoperative supervision makes such operations of little use because of the problems of monitoring and treating patients for subsequent rejection. The ability of topically applied CyA to inhibit corneal graft rejection in a rabbit model, which is a much closer analogue of the clinical situation in man than previous models, means that there may be an important role for topically administered CyA. Furthermore, these observations indicate that at least a substantial proportion of the events in sensitization induced by corneal transplantation that can be inhibited by CyA occur locally in the ocular tissue. This strengthens the concept of the eye as an immunologically competent organ (Jones, 1973).

We wish to thank Dr J. F. Borel of Sandoz Ltd for supplying the cyclosporin A and his invaluable advice, Professor A. Garner for examining and interpreting the histological material, Dr W. Ng for his technical assistance and the Governors of Moorfields Eye Hospital and the Trustees of the Pocklington Eye Transplantation Research Unit for their generous financial support.

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ELSEVIER

Pharmaceutica Acta Helvetiae 73 (1999) 293–301

PHARMACEUTICA ACTA HELVETIAE

Physicochemical properties and hemolytic effect of different lipid emulsion formulations using a mixture of emulsifiers

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Abstract

The purpose of this study was to elucidate the influence of ternary mixtures of different emulsifiers on the physicochemical properties of lipid emulsions by the aid of simplex lattice design with constraints. The physicochemical properties and the stability during the autoclaving process, as a consequent result, were investigated by examining the changes in particle size, zeta potential, and cloud point. The emulsifiers chosen were lecithin, Synperonic F68 and Tween 80. In addition, their potential for hemolysis was evaluated as it may serve as an in vitro toxicity screening method. The formulations with only Tween 80 as an emulsifier showed a noticeable change in the particle size during autoclaving as well as a remarkable erythrocyte membrane damage. In contrast, phospholipids and Synperonic F68 formulations displayed good stability during autoclaving and showed almost no hemolytic activity. Moreover, mixing Tween 80 with either lecithin or Synperonic F68 improved the stability of these formulations during the autoclaving process. Simultaneously, this led to a remarkable decrease in the hemolytic effect. This observation could be partially correlated with the cloud point, which increased by adding lecithin and was less influenced by adding Synperonic F68. This in turn resulted in an increase in the surface charge, when lecithin was added. This results in a higher resistance of the emulsifier complex to dehydration producing a more stable emulsifier film around the oil droplets. These factors inhibited the flocculation of the emulsifiers and could hinder the coalescence of the oil droplets during the autoclaving process. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Lipid emulsion; Emulsifier mixture; Physicochemical properties; Hemolytic effect; Cloud point; Simplex lattice design

1. Introduction

The emulsifying properties of surface-active agents are of great importance in the formulation of emulsions for pharmaceutical, nutritional and cosmetic applications.

Phospholipids obtained from egg yolk or soybeans are generally used in the formulation of emulsions for intravenous administration, including those for use in parenteral nutrition (Hailer and Wolfram, 1986; Sayeed et al., 1987) and as drug delivery systems (Hashida et al., 1977; Collins-Cold et al., 1990).

Recently, a mixture of phospholipids with non-ionic surfactant (Synperonic F68) was suggested for use in parenteral application (Benita et al., 1986), as it showed improved stability in comparison to emulsions stabilized only with phospholipids (Levy and Benita, 1989; Magdassi and Siman-Tov, 1990). Although this combination led to an improvement in the emulsion stability, there have been only few quantitative studies dealing with this phenomenon (Weingarten et al., 1991).

The non-ionic surfactant, polyoxyethylene-(20)-sorbitan monooleate (Tween 80), is used as an emulsifier in pharmaceutical preparations and it was suggested for use in parenteral applications (Kellner et al., 1951). It is well known, however, that emulsions stabilized with only Tween 80 displayed a great change in the particle size during

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autoclaving, and limit its application in these systems (Yamaguchi et al., 1995; Jumaa and Müller, 1998a). It is well established that the particle size distribution of parenteral fat emulsions is a critical factor for patient safety because larger particles may cause embolism (Jeppsson et al., 1976). Furthermore, the physical stability of these emulsions to terminal autoclaving is a prerequisite for the development of stable, sterile formulations (Chaturvedi et al., 1992).

Moreover, Tween 80 has a typical emulsifier structure, whereas Synperonic 68 is a block copolymer with a special structure (Bahadur and Riess, 1991), and this may lead to a different type of interaction with phospholipids (Weingarten et al., 1991; Jumaa and Müller, 1998b).

Therefore, the aim of this study was to investigate the interaction (in a ternary mixture) between Synperonic F68 and Tween 80, as non-ionic emulsifiers, with phospholipids and subsequently to determine their influence on the physicochemical properties and the hemolytic behaviour of the lipid emulsions (LE). A further aim was to find a suitable formulation for parenteral application with improved stability by making use of the steric stability of the non-ionic surfactants and simultaneously to obtain a negligible hemolytic activity. This would result in a minimization of the side effects of these systems, which may be of clinical relevance.

2. Materials and methods

2.1. Materials

Lipoid S75 (S75) and lysophosphatidylcholine were obtained from Lipoid (Ludwigshafen, Germany). Lipoid S75 was isolated from soybean and contained, according to the manufacturer, a minimum of 70% phosphatidylcholine, 10% phosphatidylethanolamine and 1.7% lysophosphatidylcholine. Purified castor oil was purchased from Henry Lamotte (Bremen, Germany). Medium-chain triglycerides (MCT) were obtained from Hüls (Witten/Ruhr, Germany). Polyoxyethylene-(20)-sorbitan monooleate (Tween 80) and polyoxyethylene–polyoxypropylene copolymer surfactant (Synperonic F68) were supplied by ICI (Atlas Chemie, Gemany). Sorbitol was purchased from Merck (Darmstadt, Germany). Diethyleneglycol-monobutylether (butyl diglycol) was obtained from Aldrich (Steinheim, Germany). Double distilled water was used and all other chemicals were of reagent grade.

2.2. Preparation of LE

The emulsions containing 20% oil phase were prepared as follows: Synperonic F68 and Tween 80 were placed in water, while S75 was dissolved in the oil phase. The oil

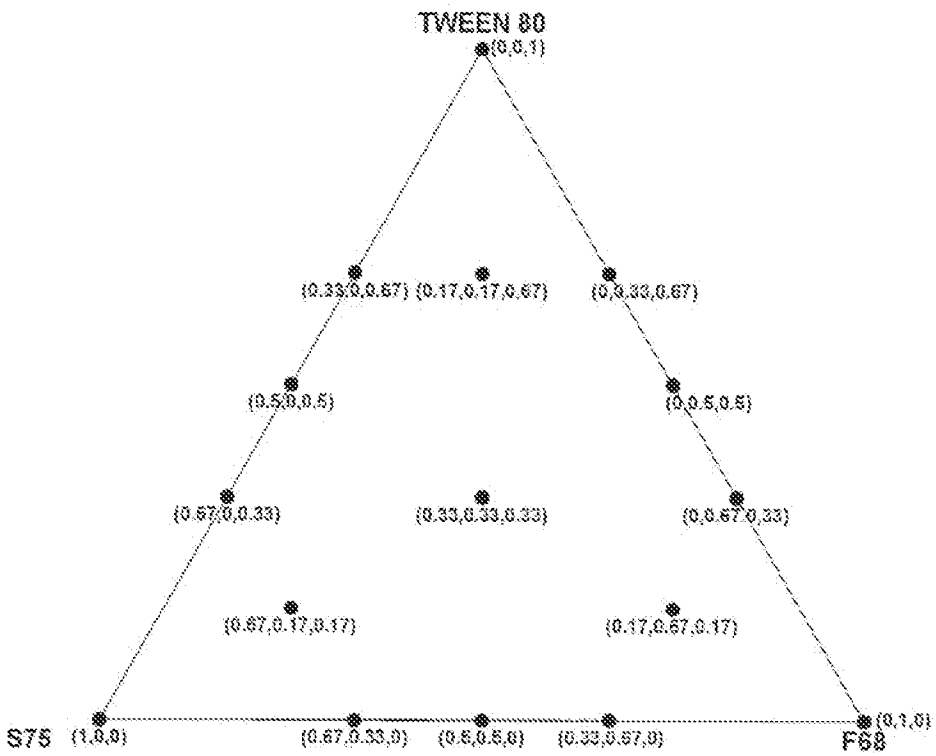

Fig. 1. Contour plot of the composition of the emulsifier mixtures (according to a simplex lattice design).

mixture and the 5% aqueous solution of sorbitol (for adjustment to isotonicity) were heated separately to about 50–55°C. The oil phase was added to the water phase and this mixture was pre-emulsified using an Ultra-Turrax T25 (Jahnke and Kunkel, Staufen, Germany) running at 8000 rpm for 3 min.

Final emulsification was carried out by passing 40 ml of the coarse emulsion through a high pressure homogenizer (Micron Lab 40, APV Gaulin, Lübeck, Germany) eight times at a pressure of 20 MPa. The homogenization was performed at a temperature of 40°C.

The pH was adjusted before autoclaving to about 8 using 0.1 N sodium hydroxide solution. The batches of emulsions were filled in 15 ml vials, sealed and sterilized using a steam autoclave (K15T, Keller, Weinheim, Germany) at 121°C for 15 min.

2.3. Measurements

2.3.1. Particle size analysis

The mean diameter [mean particle size (m.p.s.)] of the bulk population was determined by photon correlation spectroscopy (PCS) covering the size range of 5 nm to approximately 3 μm (Malvern spectrometer RR 102, Malvern, UK, with Helium-Neon laser $\lambda = 632.8$ nm, Siemens, Germany). For size analysis approximately 1 μl fat emulsion was added to 1 ml distilled water in order to obtain the optimum scattering intensity.

Larger particles were detected by laser diffraction analyser LDA (Helos, Sympatec, Clausthal-Zellerfeld, Germany) at a focal length of 20 mm, corresponding to a measurement range of 0.18–35 μm. The emulsions were characterized by Dmax and the D50 and D99 quantiles of

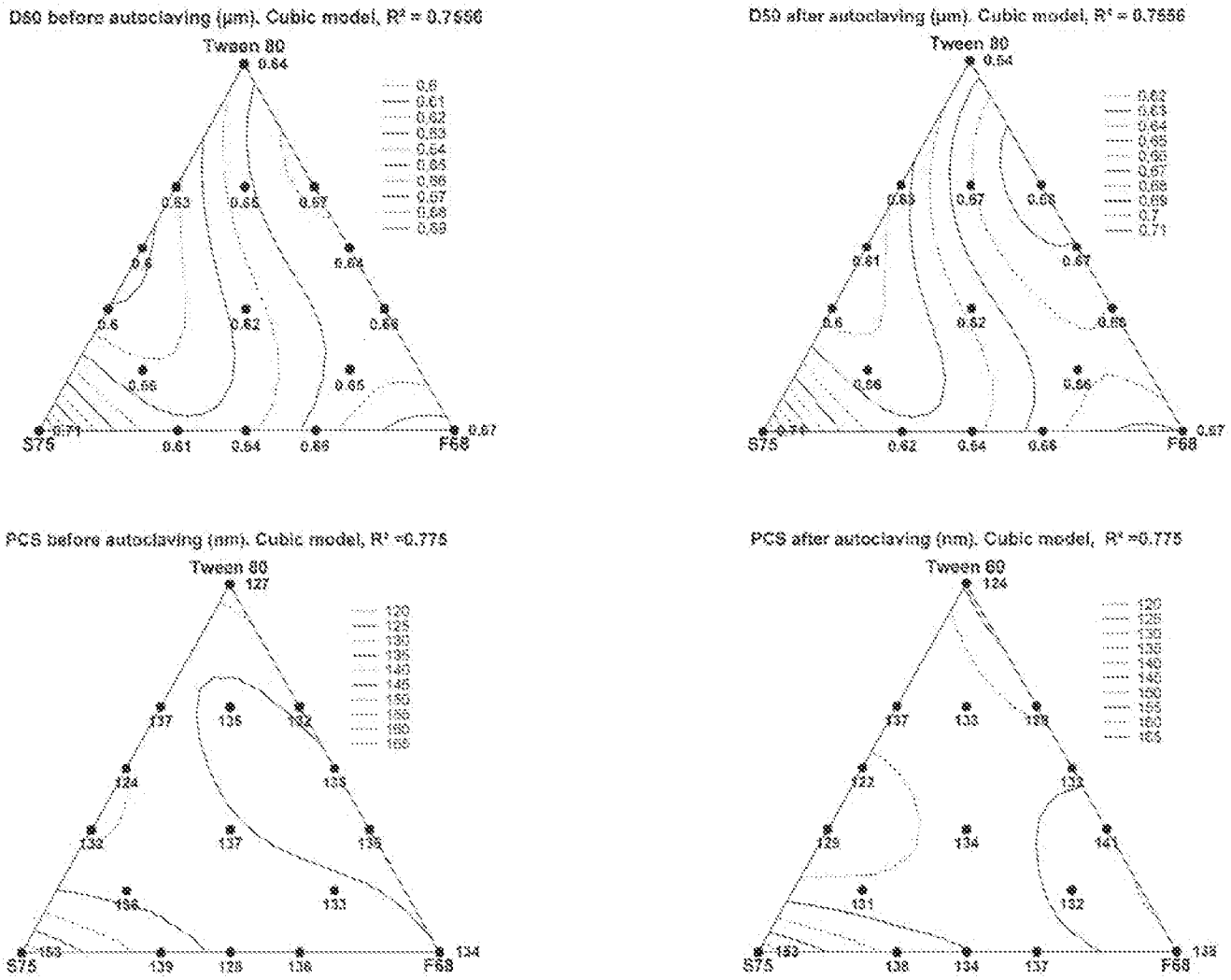

Fig. 2. Contour plot of D50 and PCS values before and after autoclaving.

the volumetric distribution (that means 50%, 99% or all of the particles (D99) were below the given size). Additionally, the specific surface area (S, m^2/ml) was calculated from the volume data.

2.3.2. Zeta (ζ) potential

The surface charge (ζ potential) was measured using a ZetaSizer 3 (Malvern Instruments, Malvern, UK). The electrolyte solution used for dilution consisted of double distilled water with a conductivity of 50 µS/cm adjusted by NaCl (0.5 mmol/l). 500 µl of each emulsion formulation was diluted with 20 ml electrolyte solution.

2.3.3. Determination of cloud point

Phospholipids are not soluble in water. In order to determine the cloud point, however, the emulsifiers need to be dissolved in water. For this reason, lysophosphatidylcholine was used as a substitute for phospholipids, which to some extent has the same properties as phospholipids. The cloud point and the phase inversion of the micellar emulsifier solutions (1% w/w) were determined by heating the solutions at a rate of 1°C per minute and noting the onset of turbidity. This was carried out in water solutions, in 20% saline solutions (w/w NaCl solutions) or in 25% butyl diglycol solutions to reduce the cloud point below 100°C. The same behaviour was observed in saline and butyl diglycol solutions and, therefore, only the results of saline solutions are shown. The temperature at which the solution started to become hazy was recorded. After heating to an additional 3°C, the solution was gradually cooled off and the temperature at which the solution became clear was noted. The average temperature of these two values was the cloud point of the micellar solution. The final results were recorded as the mean of three determinations.

2.3.4. Hemolysis studies

The method for detecting hemolysis has been described previously (Bock and Müller, 1994). Erythrocytes were isolated from heparinized human blood by centrifugation (1000 × g for 5 min). The supernatant was discarded and the erythrocytes were resuspended in isotonic phosphate buffer, pH 7.4 (PBS) to remove debris and serum protein. After centrifugation and discarding of the supernatant, the washing steps were repeated three times. An erythrocyte stock dispersion with a fixed concentration of hemoglobin was prepared with buffer (3 parts centrifuged erythrocytes plus 11 parts PBS), which yielded an absorption of approx. 2% after total hemolysis in the assay (control = 100% hemolysis). The hemoglobin concentration in the stock dispersion was about 4 mM. A 100 µl aliquot of the erythrocyte stock dispersion was added per milliliter of sample. After incubation while shaking, debris and intact erythrocytes were removed by centrifugation (750 g for 3 min) and 100 µl of the supernatant was dissolved in 2 ml of an ethanol/HCL mixture [39 parts of 99% ethanol (v/v) and 1 part of 37% hydrochloric acid (w/v)]. This mixture dissolved all components and avoided precipitation of hemoglobin. The absorption was determined at 398 nm by photometric monitoring against a blank sample. One hundred percent hemolysis was obtained after total hemolysis in double-distilled water.

2.3.5. Experimental design

The influence of the emulsifier mixtures on the emulsion properties was studied using a simplex lattice design with constraints. The ratio of water (78%), surfactant (2%) and oil phase (20%) in the formulation was kept constant. The composition of the emulsifier mixtures was varied according to Fig. 1. Each of the 16 formulations of a trial was produced three times in order to estimate the precision of the production method. The m.p.s. from PCS, Dmax and the 50% and 99% quantiles of the particle size distribution (LDA values) were used as response values. These were determined before and after autoclaving. At the same time, hemolysis and the cloud point (in water and in 20% saline solution or in 25% butyl diglycol solution) were studied. Additionally, the ζ potential of the emulsions was determined before and after autoclaving.

2.3.6. Statistical evaluation

The results were evaluated using the program Statistica (Version 5, StatSoft, Tulsa, USA). The results were analyzed according to ternary mixture models of increasing

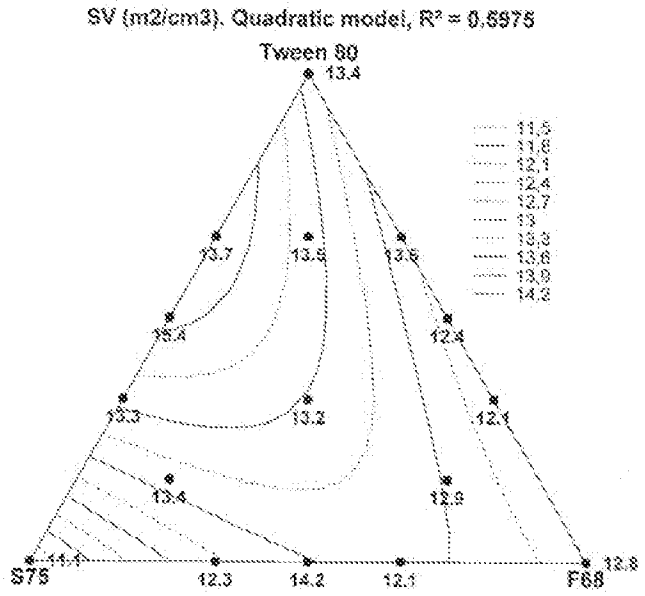

Fig. 3. Contour plot of specific surface area (S_v).

complexity: linear, quadratic, special cubic and cubic model (Cornell, 1990). All four models were fitted stepwise to the 48 results for each response value. The simplest model describing the results adequately was chosen for graphical representation. Model selection was based upon the significant contribution of the coefficient for the different models.

3. Results and discussion

3.1. Physicochemical properties

The effects of the ternary mixture on the particle size and their subsequent influence on the particle size during the autoclaving process were investigated.

Fig. 2 shows the surface plot of the D50 (the mean value obtained from laser diffraction analyzer) and the m.p.s (from PCS). The addition of the non-ionic surfactants led to a decrease in the D50 and PCS values especially in the case of formulations consisting of phospholipids and non-ionic surfactants in a ratio 1:1. This was additionally reflected by the specific area (S_v) obtained from LDA, which increased with the addition of the non-ionic surfactants, due to the decrease in particle size (Fig. 3). This could be explained on the basis of the formation of a closely-packed mixed film by intercalation of the non-ionic surfactant molecules between the phospholipid monolayers (Weingarten et al., 1991; Jumaa and Müller, 1998b). Moreover, there was no significant change in the mean values (m.p.s and D50 for all the formulations) before and after autoclaving (Fig. 2).

The values of the large particles (Fig. 4) show the same pattern that the addition of the non-ionic surfactants leads to a decrease in the particle size, with the exception of the formulations containing more than 67% Tween 80 which

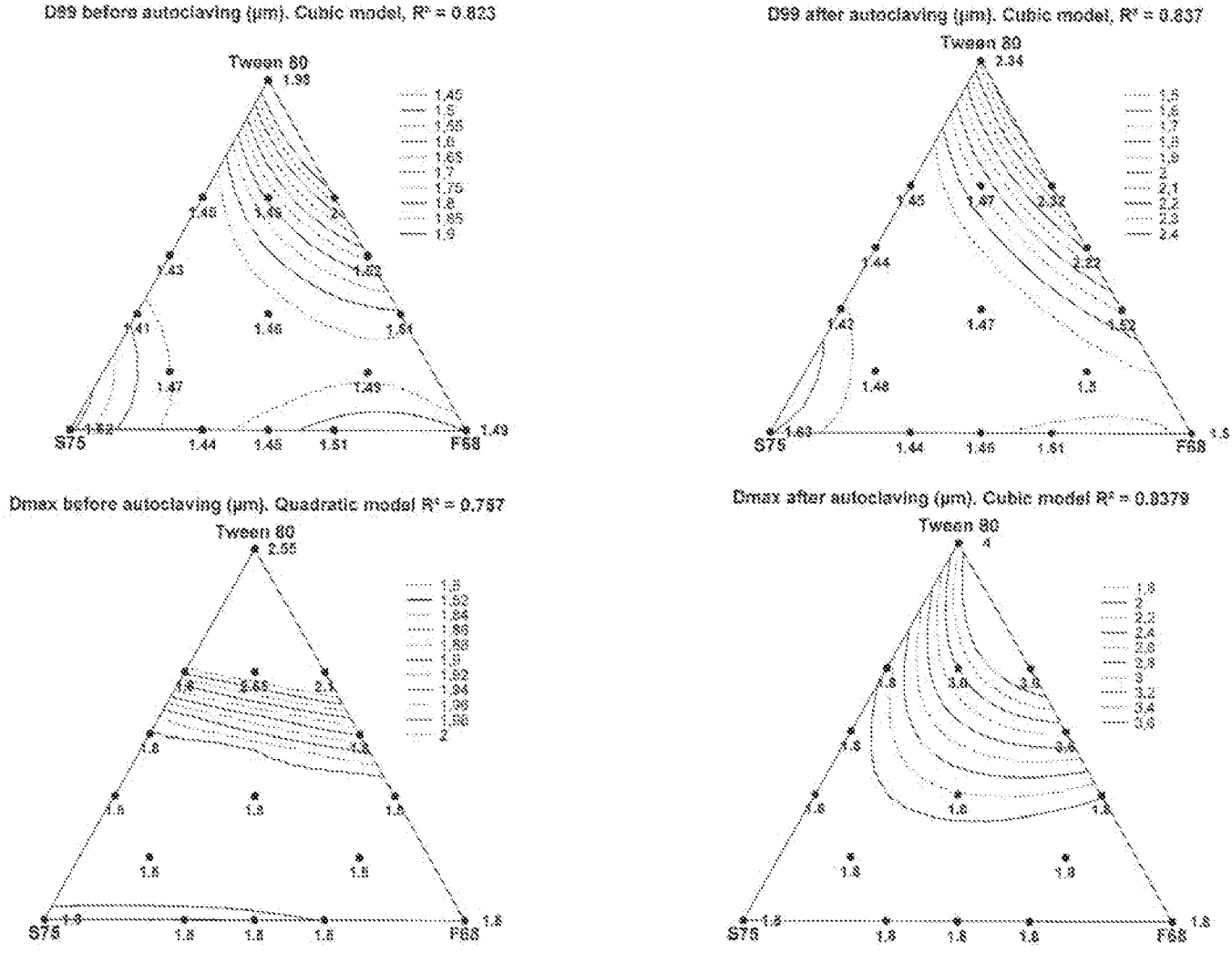

Fig. 4. Contour plot of D99 and Dmax values before and after autoclaving.

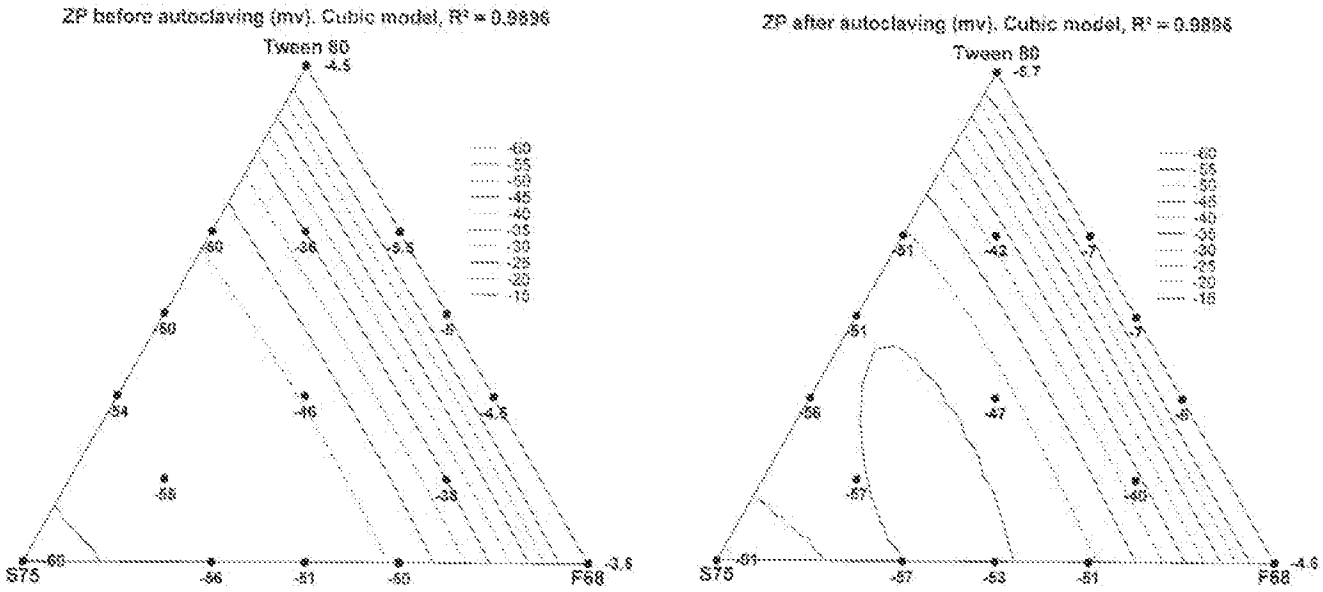

Fig. 5. Contour plot of zeta potential (ZP) values before and after autoclaving.

again showed larger particles. However, the mean diameter of these formulations was decreased as reflected in Fig. 2 and the S_v values. Therefore, the formation of these large particles could be attributed to the homogenization conditions. A pressure higher than optimum leads to a decrease in the m.p.s. and simultaneously to an increase in the particle size distribution (Tamakura et al., 1983; Junan and Müller, 1998c). Moreover, not all formulations showed good stability during autoclaving. Fig. 4 shows a remarkable change in the D99 and Dmax of some formulations after autoclaving. These changes were observed only in formulations with a large amount of Tween 80 (up to 50% in the case of Tween 80–F68 and up to 67% in the case of Tween 80–S75).

3.2. ζ Potential

As shown in Fig. 5, the addition of the non-ionic surfactant led to a small decrease in the surface charge of the droplets. Moreover, no significant change in the ζ potential before and after autoclaving was observed.

Table 1
The cloud points of the ternary mixtures of the emulsifiers (in water)[a]

Tween 80	F68	S75	Cloud point (°C)
0	1	0	> 100
1	0	0	73
0.5	0.5	0	95
0.67	0.33	0	89

[a]All other points in the ternary mixture have a cloud point higher than 100°C in water, therefore, they are not listed here.

3.3. Cloud point and phase inversion temperature

It has previously been established that emulsion formulations, containing an emulsifier with a high cloud point, were more resistant to coalescence during the autoclaving process (Junan and Müller, 1998a). Therefore, the cloud points of the ternary emulsifier mixtures were measured both in aqueous solution and in 20% (w/w) NaCl solution, with the aim of decreasing the cloud points (the cloud point of the phospholipid formulation in 20% saline solution was still above 100°C). The results are shown in Table 1 and Fig. 6, respectively. It could be deduced from these

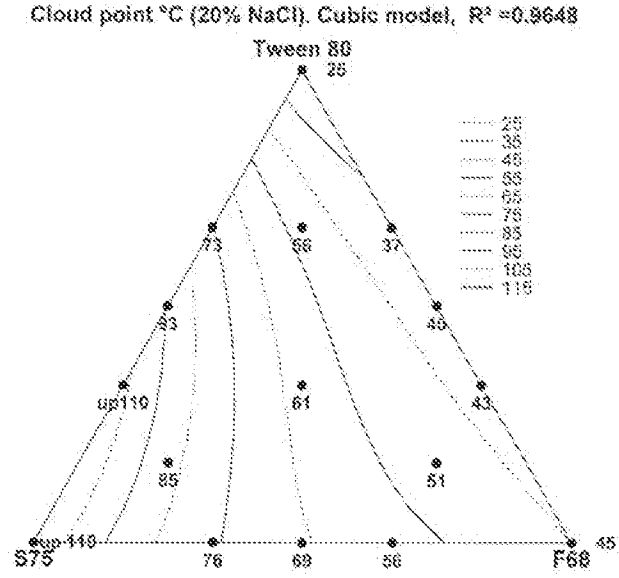

Fig. 6. Contour plot of cloud point (°C) in 20% NaCl solution (w/w).

Table 2
The effect of NaCl (A) and butyl diglycol (B) on the cloud point of F68 and their consequent effect on the particle sizes before (a) and after (b) autoclaving

A: The effect of NaCl

NaCl amount (g%)	Cloud point (°C)	D50 (μm)		D99 (μm)		Dmax (μm)	
		a	b	a	b	a	b
0	up 100	0.67	0.68	1.49	1.50	1.8	1.8
0.5	up 100	0.67	0.68	1.5	1.51	1.8	1.8
1	up 100	0.66	0.68	1.50	1.52	1.8	1.8
2	100	0.66	0.73	1.49	1.60	1.8	1.8
3	96	0.66	0.74	1.50	1.77	1.8	3.6

mean values of three measurements

B: The effect of butyl diglycol

Butyl diglycol amount (g%)	Cloud point (°C)	D50 (μm)		D99 (μm)		Dmax (μm)	
		a	b	a	b	a	b
0	up 100	0.66	0.67	1.48	1.49	1.8	1.8
3	up 100	0.66	0.67	1.48	1.49	1.8	1.8
5	≈ 100	0.65	0.71	1.47	1.59	1.8	1.8
6	98	0.65	0.79	1.47	1.77	1.8	3.6
8	92	0.66	1.38	1.47	2.81	1.8	4.2

results that Tween 80 had the lowest cloud point. Moreover, the addition of S75 or F68 led to an increase in this value and the effect of the phospholipids was more significant than F68.

When comparing the cloud point results with the D99 and Dmax results, before and after autoclaving (Fig. 4), it might be concluded that the formulations with a cloud point higher than 100°C exhibit negligible changes in their physicochemical properties, whereas formulations with lower cloud points undergo noticeable changes.

To support this explanation, the formulation with F68 (2% of F68 only) which showed a satisfactory stability during autoclaving, was suggested for further investigation. F68 non-ionic surfactant was chosen to avoid charge interactions with the added substance (NaCl and butyl diglycol). Phospholipids often show such interactions, resulting in a reduction of the ζ potential or in a disturbance in the lamellar structure of phospholipids (Rydhag, 1979; Washington, 1990). The investigation was carried out using different concentrations of sodium chloride (as ionic

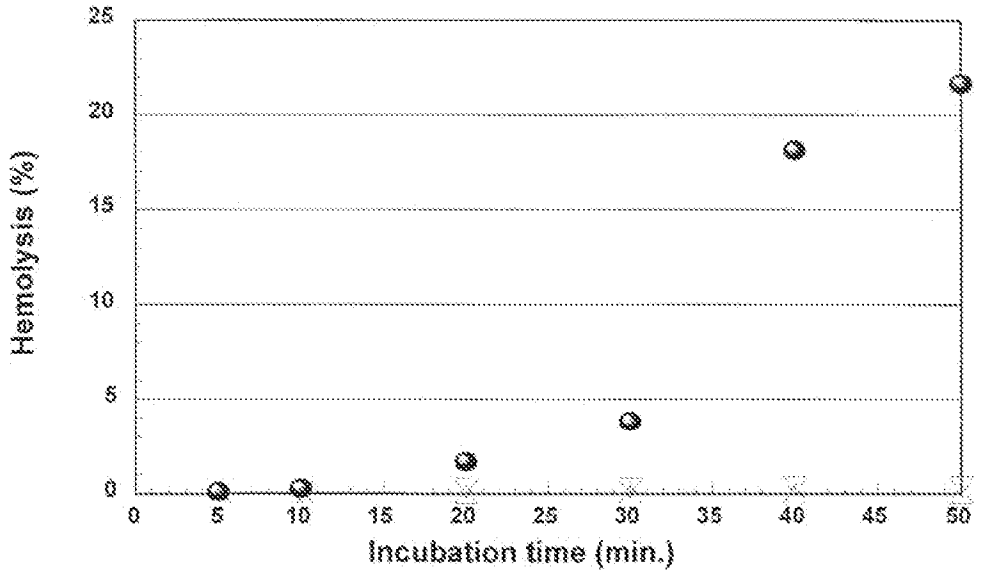

Fig. 7. The effect of the incubation time on the hemolytic activity of the formulations containing 2% of S75 (▿), F68 (△) or Tween 80 (●) [w/w].

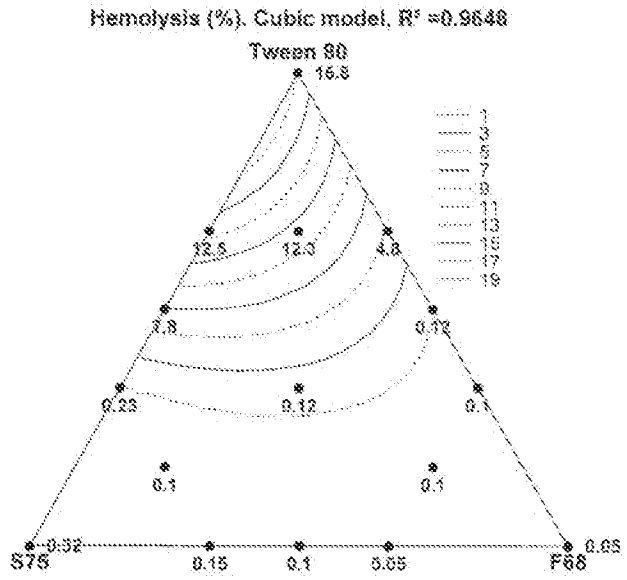

Fig. 8. Contour plot of the hemolytic activity of the different formulations.

substance) or butyl diglycol (as a non-ionic substance), which have been deemed suitable for such investigations (Müller, 1978). The results are listed in Table 2. It could clearly be deduced that a reduction of the cloud point of F68 below 100°C results in a great change in the particle size after autoclaving (especially Dmax). This suggests that formulations with a high cloud point are more resistant to dehydration at high temperatures occurring in the autoclaving process. This results in a more stable emulsifier film, which can prevent the coalescence of oil droplets during autoclaving (Clint, 1988). Therefore, no changes of particle size are observed. In contrast to this, formulations with emulsifiers and/or with emulsifier mixtures, which have a low cloud point, precipitate at the sterilization temperature leading to a breakdown of the film around the oil droplets and resulting in the coalescence of the droplets.

3.4. Hemolytic activity

The hemolytic activity has been suggested as a toxicity screen in vitro, serving as a simple and reliable measure for estimating the membrane damage caused by formulations in vivo.

It is well established that erythrocytes are affected by the incubation time (El-Hariri et al., 1992). Therefore, the erythrocyte membrane damage induced by the formulations containing S75, F68 and Tween 80, respectively, was studied after different incubation times. The results are shown in Fig. 7. From these results it can be seen that the S75 and F68 formulations do not show any hemolytic activity after 50 min, whereas the Tween 80 formulation shows hemolytic activity beginning after 20 min and increasing with time. However, no significant change between 40 and 50 min was observed. Hence an incubation time of 45 min was chosen for the investigation of the ternary emulsifier mixture.

As shown in Fig. 8, the addition of S75 or F68 led to a remarkable decrease in the hemolytic activity induced by Tween 80 with F68 having a stronger effect than S75. For example, the formulation containing F68 and Tween 80, (50/50) led to negligible hemolysis, whereas the same formulation with S75 instead of F68 exhibited about 7% hemolysis. This could be related to the ability of F68 to form an additional layer around the emulsifier mixed-film, reducing the direct contact of Tween 80 with the cell membrane, leading to a reduction in toxicity (Forster et al., 1988; Weingarten et al., 1991).

4. Conclusion

It has been demonstrated that the addition of an emulsifier with a high cloud point (phospholipids or F68) results in an increase in the cloud point of the emulsifier mixture. This leads to higher resistance of the emulsifier film against breakdown during the autoclaving process and stabilizes the emulsions.

It could also be deduced that the addition of the non-ionic surfactants leads to a decrease in the particle size and at the same time the steric stability of these emulsifiers could be used. However, Tween 80 showed no extra advantages in comparison to F68, on the contrary, Tween 80 showed a lower cloud point and remarkable hemolytic activity.

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The Effect on the Cornea of Alpha Cyclodextrin Vehicle for Cyclosporin Eye Drops

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CYCLOSPORIN (CYA), a cyclic undecapeptide of fungal origin, has been shown to be a useful immunosuppressive compound and is used widely in organ transplantation. Its application in corneal transplantation has been rather limited because of its insolubility in water. In a previous study,[1] we tested several solvents as possible vehicles for CYA, including peanut oil, palm oil, polyoxyethylene castor oil, medium-chain triglyceride emulsion (MCT), alpha cyclodextrin (α-CD), and glyceryl dicaprylate (GDC), in rats and rabbits. CYA and α-CD had the greatest penetrating ability in the cornea.

We then investigated the effect on the cornea of different concentrations of α-CD and CYA.

MATERIALS AND METHODS

We tested four solutions of α-CD that had different concentrations with maximum solubility of CYA: 0.075, 0.025, 0.009, and 0.003% CYA combined with 80, 40, 20, and 10 mg/mL α-CD. The drugs and normal saline solution were instilled 10 times every 30 minutes on three rabbits. Ultrasonic pachymetry and the Draize test were performed before and after application of the eye drops. Thirty minutes after the last eye drops, the rabbits were sacrificed with an overdose of anesthetics. After harvesting the corneas, one-half was examined under the light microscope, and the other half with the transmission and scanning electron microscope. CYA penetration into the corneal tissue was investigated using three rabbits. The drugs were instilled four times every 2 hours. The corneas were excised from the eyeball, and epithelial cells were scraped off by spatula. CYA concentration in the cornea without epithelium was measured by means of radioimmunoassay.

RESULTS

Ultrasonic pachymetry indicated that the difference in the central corneal thickness before and after application of the drugs was not statistically significant (Table 1). The Draize test for cornea, conjunctiva, and iris by the judgment of Kay and Calandra revealed that 0.075% CYA (α-CD[80 mg/mL]) and 0.025% CYA (α-CD[40 mg/mL]) were practically nonirritating and that 0.003% CYA (α-CD[10mg/mL]) was completely nonirritating. Thick sections of corneal button stained with toluidine blue appeared normal in four different concentrations of CYA (Fig 1). However, using a scanning electron microscope, we found an increased number of craters on the superficial epithlial cells in 0.075% CYA eye drops given ten times every 30 minutes. With 0.025% or lower concentrations of CYA, the same number of craters as that found in the saline eye drops group, was found (Fig 2). With the transmission electron microscope, the findings were unremarkable except for a diminution in number of microvilli on the epithelial surface associated with 0.075% CYA. Most of microvilli were normal in 0.025% or lower concentrations of CYA (Fig 3, A and B).

Concentrations of CYA in corneal tissue after topical administration of four different concentrations of CYA eye drops are shown in Fig 4. We measured the concentration of CYA in the cornea without epithelium using RIA methods. The median levels with standard errors of the median are 4886 ± 706 ng/g in 0.075% CYA, 4133 ± 467 ng/g in 0.025% CYA, 1580 ± 621 ng/g in 0.009% CYA, and 1280 ± 296 ng/g in 0.003% CYA.

DISCUSSION

CYA was introduced as a powerful immunosuppressive agent for clinical organ transplantation. In ophthalmological applications, it has been demonstrated that administration of CYA by eye drops,[2-4] and subconjunctival[7] or retrobulbar injection[8] prolongs corneal allograft survival in rabbits. Hoffmann and associates[9] reported that topical administration of 2% CYA in castor oil suppressed graft rejection clinically. However, a major obstacle in the application of CYA in ophthalmic preparations is its insolubility in water. In our previous study,[1] the results of histological examination after topical administration of several lipophilic vehicles on rat corneas indicated that significant corneal edema was associated with castor oil and palm oil compared with MCT, α-CD, GDC, and peanut oil. In our study,[1] MCT, α-CD, GDC, and peanut oil combined with different concentrations of CYA were instilled on rabbit eyes 10 times every 30 minutes. The Draize test demonstrated that all drops were minimally irritating. Electron microscopic examination revealed superficial epithelial toxicity, such as loss of microvilli and microerosion in both α-CD (80 mg/mL) and GDC. CYA level in the cornea without epithelial layer was mea-

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Table 1. Pachymetry of Rabbit Cornea Before and After Topical Administration of Four Different Concentrations of Cyclosporin

Concentration CYA (%)	Concentration α-CD (mg/mL)	No.	Before (mm)	After (mm)	Difference (mm)	Significance
0.075	80.0	3	0.348 ± 0.012	0.346 ± 0.011	−0.003 ± 0.005	NS
0.025	40.0	3	0.346 ± 0.014	0.343 ± 0.007	+0.003 ± 0.009	NS
0.009	20.0	3	0.356 ± 0.008	0.338 ± 0.004	−0.002 ± 0.010	NS
0.003	10.0	3	0.354 ± 0.008	0.342 ± 0.008	−0.012 ± 0.001	NS
Control (saline solution)		8	0.358 ± 0.02	0.351 ± 0.01	−0.01 ± 0.01	

sured with RIA. α-CD had the highest level of concentration, and GDC the lowest.

With these facts in mind, we investigated the acceptable concentration of α-CD that would produce the least corneal toxicity. We evaluated four different concentrations of α-CD with maxium solubility of CYA. Typical adminstration of 0.025% CYA (40 mg/mL) caused minimal irritation as measured by the Draize test, with little superficial damage. The concentration of CYA in the cornea was about 5 to 10 times higher than that with the use of 10% CYA ointment,[9] epibulbar administration of 5% CYA,[11] and with our previous several vehicles associated with CYA.[3] This result suggests that 0.025% may be considered an acceptable concentration for CYA eye drops and can be effective in suppressing rejection of corneal grafts.

SUMMARY

We tested four different combinations of α-CD and CYA: 0.075% and 80 mg/mL α-CD, 0.025% CYA and 40 mg/mL α-CD, 0.009% CYA and 20 mg/mL α-CD, and 0.003% CYA and 10 mg/mL α-CD. We found that 0.025% CYA

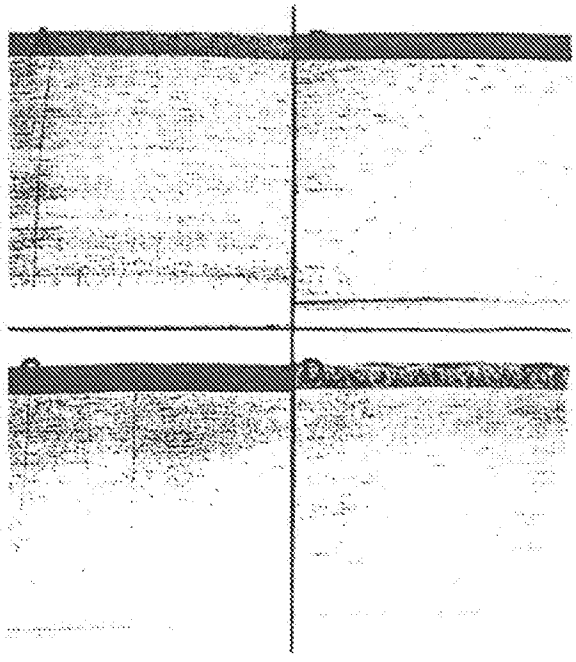

Fig 1. Light micrograph of rabbit cornea after different administrations of CYA. (A) 0.075% CYA (α-CD 80 mg/mL). (B) 0.025% CYA (α-CD 40 mg/mL). (C) 0.009% CYA (α-CD 20 mg/mL). (D) 0.003% CYA (α-CD 10 mg/mL).

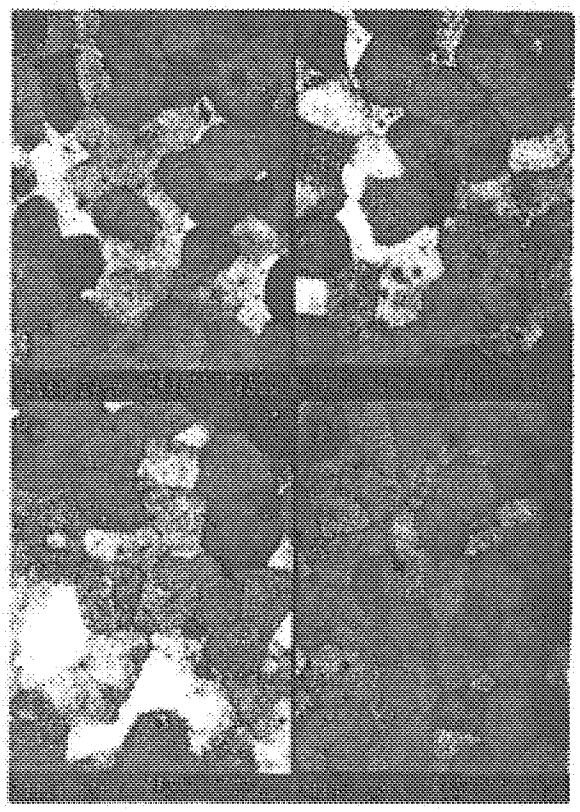

Fig 2. SEM of rabbit cornea after different administrations of CYA. (A) 0.075% CYA (α-CD 80 mg/mL). (B) 0.025% CYA (α-CD 40 mg/mL). (C) 0.009% CYA (α-CD 20 mg/mL). (D) 0.003% CYA (α-CD 10 mg/mL).

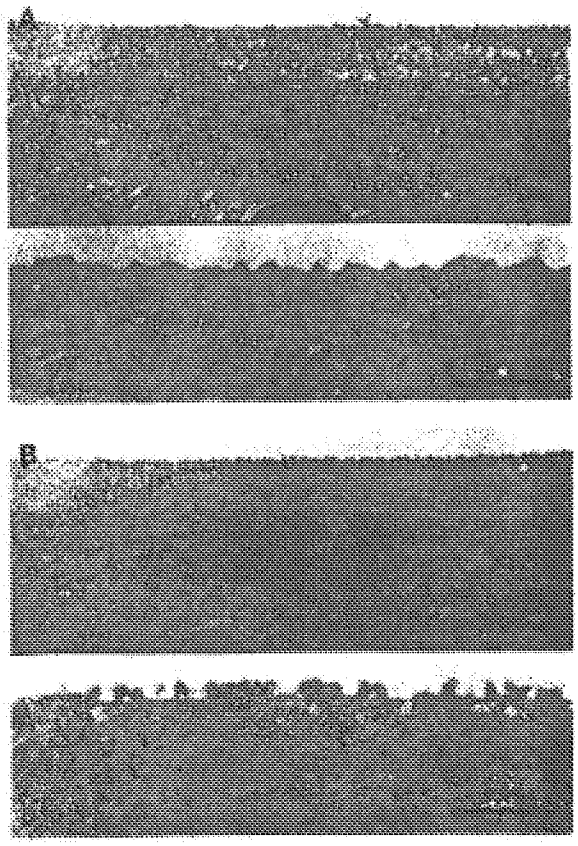

Fig 3. TEM of rabbit cornea after the administration of 0.075% CYA (A) and 0.025% CYA (B). (A) Upper 7,500×, lower 25,000×. (B) Upper 7,500×, lower 25,000×.

(α-CD|40 mg/mL]) resulted in the least corneal toxicity and penetrated in the cornea 5 to 10 times more than did lipophilic vehicle with CYA.

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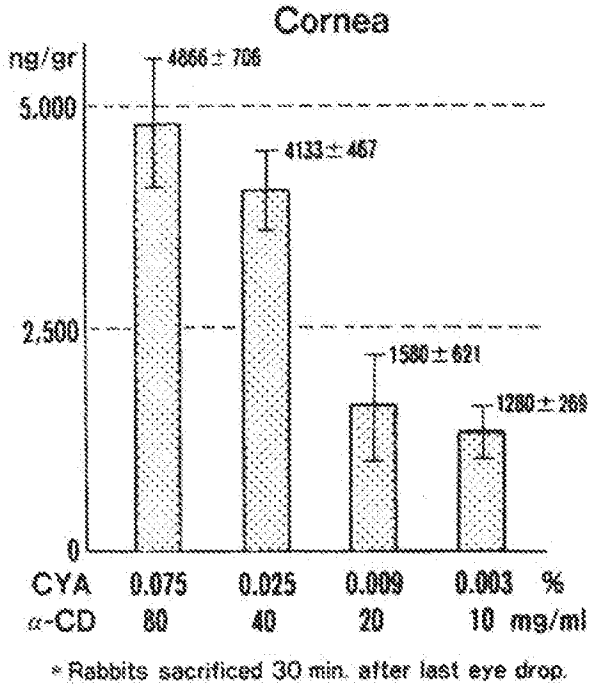

Fig 4. CYA (α-CD) concentration in the cornea measured by means of RIA.

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Penetration of Cyclosporin A into the Rabbit Cornea and Aqueous Humor after Topical Drop and Collagen Shield Administration

Ayfer Kanpolat, M D Figen Batioglu, MD Merih Yilmaz, MD Fatma Akbas, MD

We used commercially available 12-hour collagen shields to deliver cyclosporin A (CsA) to the cornea and aqueous humor in rabbit eyes. Six New Zealand white rabbits were divided into three groups. The first group (four eyes) received 6 mg of CsA in castor oil and the second group (four eyes) received 6 mg of CsA in olive oil applied as topical drops to rabbit eyes within 12 hours. In the third group (four eyes) 12-hour collagen shields soaked in 6 mg CsA in olive oil were applied to rabbit eyes. The amount of CsA in corneal and aqueous samples from eyes treated with CsA castor oil and CsA olive oil were compared with each other and with collagen shield treated eyes. CsA concentrations were measured by radioimmunoassay. After the total dose of 6 mg CsA, percentage penetration was measured as follows: CsA castor oil—0.51% in aqueous and 20.75% in cornea; CsA olive oil—0.17% in aqueous and 11.13% in cornea; and with collagen shields—0.44% in aqueous and 11.84% in cornea. These results show that the CsA levels of castor oil drops were higher than those obtained with olive oil drops. In eyes with collagen shields, CsA levels were higher than olive oil drops but nearly equal to the castor oil drops. Collagen shields may be useful as an ocular delivery system for CsA.

Introduction

The successful treatment of many diseases of the eye depends upon adequate drug delivery. An important development in drug delivery has been the utilization of collagen shields. Collagen shields have been used as bandage lenses after radial keratotomy and keratorefractive surgery, to treat corneal abrasions, and as an alternative therapy for dry eyes.[1,2] The shields are made of porcine scleral collagen and are available in 12- 24- and 72-hour dissolution rates. Their oxygen permeability is high. After placement on the eye, they become hydrated with tears, take the shape of the cornea, and are degraded by proteolytic enzymes in the tear film. In addition to their capabilities of lubricating, protecting, and accelerating reepithelialization, they are used as vehicles for enhancing the ocular penetration of various drugs.[3] Recent investigations with collagen shields demonstrate that delivery of gentamicin,[4] vancomycin,[4] tobramycin,[5] dexamethasone,[6] amphotericin B,[7] heparin,[8] and cyclosporin A[9] to the eye are increased.

Cyclosporin A (CsA) is a cyclic polypeptide of fungal origin. First reported to have immunosuppressive properties by Borel in 1976,[10] it has dramatically improved the prognosis for solid organ transplantation because of its low myelotoxicity and suppression of specific T lymphocyte function. Systemic CsA has been used to suppress rejection after kidney transplantation, as well as bone marrow, heart, and liver transplantations.[10] It has also been used to treat various autoimmune diseases such as uveitis, psoriasis, rheumatoid arthritis, myasthenia gravis, and diabetes mellitus type 1.[10] It has been used with some success to treat patients with various ocular manifestations of systemic immune disease, including Grave's ophthalmopathy,[10] corneal peripheral melting syndrome,[11] Behcet's disease,[10], and Sjögren's syndrome. Nussenblatt and colleagues[10] suggested that topical

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therapy was effective only if the serum CsA levels entered the therapeutic range of 50 to 300 ng/mL.

Delivery of therapeutic concentrations of CsA to the cornea and the anterior chamber has been difficult to accomplish because of its poor solubility in water.[9] Topical ophthalmic uses have included treatment of corneal graft rejections,[12,13] Mooren's ulcer, noninfiltrative marginal keratolysis,[14] and herpetic stromal keratitis.[15] Recently, investigations of CsA levels in the eye and various tissues after topical and systemic administrations have been made. Mosteller and coworkers[16] investigated the systemic and ocular absorption of topically applied 10% cyclosporine ointment. Ben Ezra and colleagues[17] determined the tissue levels of CsA after oral, intraperitoneal, and intravenous administrations. Reidy and associates[9] prepared collagen shields by mixing gelatinous collagen with crystalline CsA and compared them with topical eye drops.

The aim of this study was to investigate the effectiveness of commercially available collagen shields and CsA preparations and was performed to compare the penetration of CsA olive oil and castor oil forms administered as topical eye drops with each other and with commercially available collagen shields soaked in CsA olive oil.

Materials and methods

Six New Zealand white rabbits, male and female, 2,038 to 3,220 g were divided into three groups.

Eye drops: In group 1, the eye drops consisted of a commercially available intravenous form of CsA (castor oil) in a concentration of 50 mg CsA/mL (0.5 mg CsA/10µL; Sandimmune, Sandoz Pharmaceuticals). In group 2, the eye drops consisted of the oral form of CsA (olive oil) in a concentration of 100 mg CsA/mL (1 mg CsA/10 µL; Sandimmune, Sandoz Pharmaceuticals). Over 12 hours, a total of 6 mg CsA was administered as topical drops with a micropipette.

Collagen shields: Four commercially available collage shields were used (12 hours [Bio-cor; Bausch & Lomb Pharmaceutics, Inc., Clearwater, FL]). For this purpose, 60 µL (1 mg CsA/10 µL) Sandimmune® oral solution (olive oil) was dropped over collagen shields. We used CsA olive oil because it was absorbed better than CsA castor oil. Within 1.5 to 2 hours, the total dose of 6 mg CsA was absorbed by the collagen shields.

Experimental design: The six rabbits were divided into three groups. In group 1, four eyes of two rabbits received CsA castor oil applied with a micropipette (10 µL [Socorex; Switzerland]) at one drop (10 µL) per hour for 12 doses for a total of 6 mg CsA. (Every 10 µL drop contained 0.5 mg CsA [12 × 0.5 mg = 6 mg CsA].) In the second group, four eyes of two rabbits received CsA olive oil applied to the eyes with a micropipette, one drop every 2 hours for 12 hours (six times). They also received a total of 6 mg CsA (every 10 µL drop contained 1 mg CsA [6 × 1 mg = 6.0 mg CsA]). In both groups, the total dose of CsA (6 mg) was equal to the amount of CsA delivered by a single collagen shield.

Four eyes of the two rabbits in the third group were treated with CsA olive oil soaked collagen shields. Rabbits were anesthetized by intramuscular injection of 0.5 mL 2% xylazine hydrochloride (Rompun; Bayer) and 1 mL ketamine hydrochloride (Ketalar, Parke-Davis). Collagen shields containing CsA were applied directly to the corneas and rehydrated with sterile saline. The eyes were closed by lid sutures to ensure retention of the shields. The shields remained in the rabbits' eyes for 12 hours.

Twelve hours after application of either the drops or the collagen shields, the rabbits were killed with sodium pentobarbital injections given intravenously. The eight eyes to which the topical eye drops had been administered were rinsed with sterile saline. Aqueous humor was collected with a sterile 25 gauge needle attached to a 2 mL syringe. The anterior chamber was entered 1 mm away from the limbal margin of the cornea, and 0.2 to 0.5 mL of aqueous humor was aspirated. The corneas were removed by 8.5 mm trephine. After rinsing them with sterile saline, the corneas were minced with a razor blade and transferred to a 1 mL volume of methanol. They were left at 4°C overnight and centrifuged at 2,000 revolutions per minute for 10 minutes.

When the four eyes to which the collagen shields were applied were opened, no shields were found on the corneas. Small fragments of collagen, combined with mucus, were lodged in the fornices. Eyes were rinsed with sterile saline, and samples were obtained in the same manner as previously described.

CsA concentrations in all samples were evaluated by monoclonal antibody based radioimmunoassay (RIA). The RIA laboratory used CYCLO Trac SP 125/RIA (INCSTAR Corp., Stillwater, MN) and a gamma scintillation counter.

Results

In group 1, CsA castor oil treated eyes, the CsA concentration in the aqueous humor ranged between 22.8 ng/mL and 38.2 ng/mL, with an average of 30.6 ng/mL (Table I); the concentrations in the cornea ranged between 865 ng/mL and 1,622 ng/mL, with an average of 1,245 ng/mL (Table II). In group 2, the CsA concentrations in aqueous humor of eyes that received topical CsA olive oil drops were below levels that could be measured with RIA (< 10 ng/mL; Table I). The values obtained in the cornea ranged between 472 ng/mL and 855 ng/

TABLE I Cyclosporine concentrations in aqueous humor (ng/mL)

	Mean	SD	Range
CsA, castor oil	30.625	8.531	+22.8 – +38.2
CsA, olive oil*	10.00	0	0
Collagen shield	26.475	13.192	+14.7 – +42.8

**CsA, olive oil < 10 ng.*
SD = standard deviation

TABLE II Cyclosporine concentrations in cornea (ng/mL)

	Mean	SD	Range
CsA, castor oil	1245	426.792	+865 – +1622
CsA, olive oil	667.8	157.559	+472 – +855
Collagen shield	710.5	129.935	+589 – +857

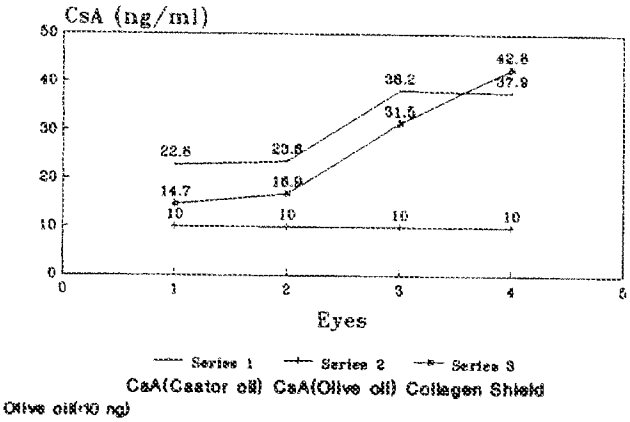

Figure 1 *Aqueous humor levels.*

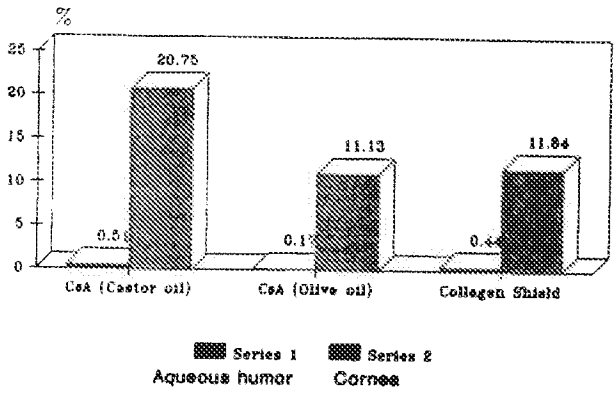

Figure 3 *Aqueous humor and cornea levels.*

mL, with an average of 667.7 ng/mL of CsA (Table II). Thus, after 12 hours, the concentrations in the aqueous humor (Figure 1) and the cornea (Figure 2) were statistically higher in the group that received topically applied castor oil CsA (aqueous humor, $P < 0.017$; cornea, $P < 0.035$, paired t test).

In group 3, CsA applied by collagen shields, aqueous humor values ranged between 14.7 ng/mL and 42.8 ng/mL, with an average of 26.5 ng/mL (Table I), and the values of the cornea ranged between 589 ng/mL and 857 ng/mL, with an average of 710.5 ng/mL (Table II). Whereas the mean aqueous humor concentrations of CsA in eyes treated with collagen shields were significantly greater than the mean concentrations of CsA in eyes treated with topically applied CsA olive oil drops, no significant difference in corneal concentrations could be demonstrated between these two groups ($P > 0.316$).

When the results obtained by using collagen shields were compared with the results of topically applied CsA castor oil drops, the concentrations were nearly equal in aqueous humor (Figure 1). According to paired t-test, no significant difference could be demonstrated ($P > 0.25$), but the corneal concentrations were higher with topically applied CsA castor oil drops (Figure 2), and this was significant ($P < 0.038$).

Figure 3 shows the penetration levels of CsA in aqueous humor and cornea with topical castor oil, olive oil drops, and olive oil soaked collagen shields as a percentage of the total dose of 6 mg.

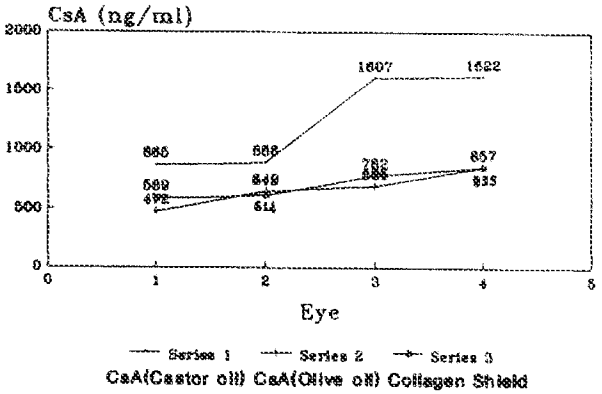

Figure 2 *Cornea levels.*

Discussion

The results of this study indicate that in rabbits topically applied CsA castor oil drops resulted in higher concentrations of the drug in the aqueous humor and cornea than when CsA olive oil drops were used. An additional finding is that a collagen shield soaked in CsA olive oil form is an equally effective means of delivering CsA to the cornea and aqueous humor as topically applied castor oil drops.

The topical application of CsA to the eye has been studied in an attempt to reduce the risk of systemic toxicity while maximizing its local therapeutic potential. Because of the hydrophobic nature of the corneal epithelium, CsA would be expected to penetrate it easily. Conversely, the stroma should be quite impermeable to the drug because it is hydrophilic. By using a 2% solution of CsA (castor oil), Wiederholdt and colleagues[18] evaluated its penetration into the different layers of rabbit eyes. Following a single dose of 10 µL of 2% CsA, a concentration of 900 to 1,400 ng/mL was detected in the cornea at 6 hours, of which 67% was found in the epithelium, 25% in the stroma, and 8% in the endothelium. Concentrations in the remaining tissues (lens, vitreous, uvea, retina) were < 45 ng/mL.[18] Kaswan[19] measured the absorption of topically administered 1% CsA in olive oil. After the total dose of 0.84 mg was given, tissue levels (cornea, anterior and posterior sclera) greater than 50 ng/mL were achieved within 1 hour, but lower levels were obtained in the retina, vitreous, ciliary body, iris, and aqueous humor.

In our study, the penetration of topically applied CsA castor oil and olive oil drops were compared with each other. After a total dose of 6 mg of topically applied CsA castor oil, 0.51% was found in aqueous humor and 20.75% in the cornea, compared with 0.17% in aqueous and 11.13% in the cornea with topical CsA olive oil drops. Thus, topical drops prepared using CsA in castor oil are more effective than CsA in olive oil.

Reidy and coworkers[9] prepared collagen shields by mixing gelatinous collagen with crystalline CsA as a means of delivering CsA to the cornea and aqueous humor in rabbit eyes.

They found that both the corneal and aqueous humor concentrations of CsA achieved with the shield delivery system were higher than those obtained with topical CsA olive oil drops. In our study, 12-hour collagen shields were soaked in CsA olive oil and applied to the eyes. CsA levels in the aqueous humor were 0.44% of the total dose, and 11.84% of the total dose was found in the cornea.

Commercially available collagen shields can be utilized as a means of delivery system for CsA and in addition to commercially available oral and intravenous forms of CsA dispensed as topical drops. Our study is the first to show the efficacy of using commercially available collagen shields as a means of delivering CsA.

The application of CsA in a collagen shield delivery system is easy to apply to patients with diseases such as Mooren's ulcer, corneal peripheral keratolysis, herpetic stromal keratitis, and corneal allograft reactions, thus minimizing systemic toxicity and maximizing local penetration.

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ERRATUM The article "Deposition of Ciprofloxacin, Prednisolone Phosphate, and Prednisolone Acetate in SeeQuence Disposable Contact Lenses," which appeared in our July 1993 issue (*CLAO J* 1993;19:166-168), failed to indicate a legal name change for one of the article's co-authors. The correct spelling of the second author's name is Ameet K. Goyal, MD. The editors apologize for this error.

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SOLID DISPERSIONS OF DRUGS IN POLYOXYETHYLENE 40 STEARATE: DISSOLUTION RATES AND PHYSICO-CHEMICAL INTERACTIONS

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Solid dispersions of drugs in water-soluble matrix materials, such as polyethylene glycol (PEG), are potentially useful dosage forms (e.g. Chiou and Riegelman, 1971). The PEG ester, polyoxyethylene 40 stearate (P40S), may offer the following advantages over PEG itself: the hydrocarbon chain, by enhancing surface activity, might subject lipophilic drug molecules to aqueous micellar solubilization and thereby promote drug dissolution (Elworthy and Lipscomb, 1968); the reduced polarity might increase the solubility of lipophilic drugs in liquid excipient and perhaps also in the solid excipient and thereby improve drug dispersion. These hypotheses are being tested by comparing solid dispersions of tolbutamide, griseofulvin and frusemide in P40S with those in PEG 2000, prepared by physical mixing, fusion or co-precipitation from ethanol. PEG 2000 and P40S have approximately the same mean molecular weight. Daily intakes of up to 25 mg of P40S per kg body weight are acceptable in food (WHO, 1974).

The solubility and dissolution rate of non-disintegrating discs of each pure drug in water increased only slightly in the presence of PEG, but increased considerably with increasing concentration of P40S in water, being an order of magnitude higher in 5% w/v P40S than in 5% w/v PEG. This difference is due to micellar solubilisation of the drugs by P40S. The critical micelle concentration of P40S is 0.014% w/v at 37°C; that of PEG is >5% w/v, if it exists.

Complete solid-liquid phase diagrams for each type of dispersion of each drug in each excipient were determined by differential thermal analysis and hot stage microscopy. These diagrams, which were of the monotectic type for PEG and mostly of the eutectic type for P40S, showed greater solubility of each drug in liquid P40S than in PEG. Microscopic examination of the solid dispersions revealed micro-crystals of each drug even when present at less than 1% w/w.

Solid discs, 13 mm in diameter, of certain of the solid dispersions were prepared by compression at 75.1 MN m^{-2}. Upon exposure to aqueous buffers at pH 1 or 7 in a simple beaker-stirrer dissolution test (Levy and Hayes, 1960), the compacts disintegrated by progressive erosion releasing flocculos of micro-crystals of the drug. The size distribution of the drug particles released from fused dispersions in P40S, were determined by Coulter counter in drug-saturated electrolyte. The mean particle size of tolbutamide decreased from >30 μm to 10 or 3 μm, whereas that of griseofulvin (micronised) was virtually unchanged.

The rate and extent of release of each drug from P40S dispersions were greater than from PEG dispersions of the same composition by weight. For example, co-precipitates containing 60% w/w of each drug disintegrated completely and the concentration of drug dissolved levelled off after less than 30 minutes from P40S dispersions but after more than 120 minutes from the corresponding PEG compacts.

Although no solid solutions of the drugs in the excipients were detected, the results support the original hypothesis in all other respects. P40S is superior to PEG 2000 in promoting dispersion in the solid, disintegration of the compacts and solubilisation of the drugs. Drug bioavailability from the dispersion systems and the effects of added disintegrants are also being studied.

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Cyclosporine A Formulation Affects Its Ocular Distribution in Rabbits

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KEY WORDS: cyclosporine A; ocular distribution; nonionic surfactant; ophthalmic formulation; eye drop; aqueous solution; autoimmune disease

INTRODUCTION

Systemic Cyclosporine A (CsA) administration averts graft rejection after organ transplantation. In the eye, CsA is also beneficial in the treatment of autoimmune diseases, uveitis, Bechet's disease, Sjögren's syndrome, keratoconjunctivis sicca, and corneal transplantation (1). It has been suggested that topical rather than systemic CsA application could also be therapeutic, without causing systemic side effects, in the treatment of ocular disease. This may be possible because much less CsA can penetrate into the bloodstream after its topical instillation in either an aqueous- or oil-based medium. It is expected that penetration from an aqueous medium will be even less than in oil because its solubility in water is much less. Various attempts were made to develop ophthalmic formulations that improve ocular CsA penetration, including an alpha-cyclodextrin vehicle (2), vegetable oils (3), liposomes (4), collagen shields (5), micro- or nanospheres (6), and oil-in-water emulsion (7). However, none of these deliver therapeutic amounts of CsA into target ocular tissues with low ocular toxicity.

In this work, we compared in rabbit ocular tissues CsA pharmacokinetics and distribution resulting from its topical application as an oil-based medium, o/w emulsion, and CsA aqueous clear solution containing a surfactant. Our results suggest that only an aqueous solution containing the nonionic surfactant MYS-40 delivers therapeutic levels of CsA.

MATERIAL AND METHODS

Chemicals

CsA was kindly donated by Novartis Pharma (Tokyo, Japan). Polyoxyethylene 20 sorbitan monooleate (Tween 80) was purchased from Kao Corporation (Tokyo, Japan). Polyoxyl 40 stearate (MYS-40) and polyoxyl 60 hydrogenated castor oil (HCO-60) were purchased from Nikko Chemical Co., Ltd (Tokyo, Japan). Pemulen® TR-2 polymeric emulsifier was supplied by BF Goodrich (Cleveland, OH). Hydroxypropyl Methylcellulose (HPMC, 65SH4000 grade) was purchased from Shin-Etsu Chemical Co., Ltd (Tokyo, Japan). Tritium labeled CsA (37 MBq/mL) was purchased from Amersham Life Science (Buckinghamshire, England). Castor oil, high-performance liquid chromatography (HPLC)-grade acetonitrile, and methanol were purchased from Nacalai Tesque, Inc. (Tokyo, Japan). All other chemicals were analytical grade and obtained from commercial sources.

CsA Assay

In the formulation studies, CsA was assayed using the modified HPLC method established by Novartis Pharma. A reversed phase YMC-Pack C_8 column (Model A-202, YMC Co., Ltd.) was used. The mobile phase was a mixture of acetonitrile:water:methanol:phosphoric acid (900:525:75:0.075). Its flow rate was 1.5 mL/min. The eluent was monitored by UV at 214 nm. The column oven temperature was maintained at 70 °C. Quantitation of peak area demonstrated excellent linearity (r^2 = 0.9999) over the CsA concentration range of interest.

Solubility Assay

Tween 80, HCO-60, and MYS-40 are nonionic surfactants that have been previously used in commercial ophthalmic products. Mixtures containing CsA and each surfactant (0.5 w/v %) were stored for 12 h to ensure equilibrium at 25 °C and then filtered through a 0.22-μm membrane filter. CsA concentrations in the filtrates were determined by HPLC. Effect of MYS-40 concentration on the solubility of CsA was determined at 25, 40, and 60 °C in the same experimental manner.

0.1 % ^{3}H-CsA Ophthalmic Formulation Preparation

^{3}H-CsA ophthalmic formulation (Aq-CsA) was prepared with the following procedure by first dissolving: CsA (0.0865 %) in a small amount of ethanol (0.1 %), and then MYS-40 (2 %) was added. The sample was well mixed until clarity returned. The CsA solution containing HPMC (0.3 w/v%), sodium dihydrogen phosphate (0.2 w/v%), and disodium EDTA (0.01 w/v%) was then adjusted to isotonicity (i.e., 287 mOsm) by the addition of sodium chloride. The pH was adjusted to 7. The needed volume of ^{3}H-CsA dissolved in an ethanol/toluene mixture was first dried in a nitrogen stream and then dissolved in the aforementioned aqueous solution containing CsA. It was stirred at 5 °C overnight to prepare Aq-^{3}H-CsA (37 MBq/mL).

To prepare a ^{3}H-CsA castor oil formulation (Oil-CsA), CsA (0.0865 %) was dissolved with stirring in a neat castor oil. ^{3}H-CsA was added in the same manner described above.

The ^{3}H-CsA o/w emulsion formulation (Em-CsA) was prepared as described (7). The oil phase consisted of CsA and castor oil. In brief, the CsA ethanolic solution was added to a mixture containing castor oil (1.25 %) and Tween 80 (1.0 %). Residual ethanol in the mixture was evaporated by a nitrogen stream. Pemulen® TR-2 (0.05 %) was dispersed in water and then mixed to obtain an o/w emulsion. This mixture was adjusted to isotonicity by the addition of glycerin and adjusted to pH 7.4. ^{3}H-CsA was added in the same manner as described previously.

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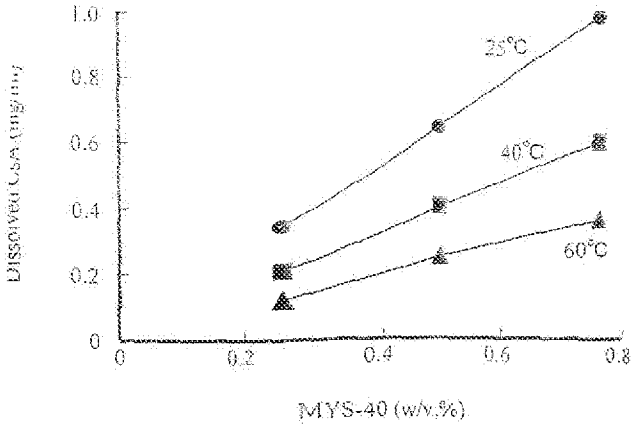

g. 1. Effects of MYS-40 concentration and temperature on the solubility of CsA in aqueous media.

cular Distribution Studies

The use and the treatment of rabbits in this study conrmed to the ARVO Statement for the Use of Animals in phthalmic and Vision Research. Male Japanese white rabits weighing between 2 to 3 kg were used. A ^{3}H-CsA ophalmic formulation (50 μL, 1850 kBq/eye) was topically intilled onto the right eyes. At 1, 4, and 24 h after instillation, he corneal epithelium, the corneal stroma and endothelium, he bulbar conjunctiva, the iris-ciliary body, the lacrimal land, the Harder gland, aqueous humor, and the choroidetina were dissected. Tissue CsA levels were calculated from he specific activity of ^{3}H-CsA and expressed as ng-eq/g tisue.

RESULTS

CsA Solubility In Ophthalmic Solutions

At 25 °C, the solubility of CsA in solutions containing one of three different surfactants (0.5 w/v %), MYS-40, HCO-60, and Tween 80, was 0.656, 0.554, and 0.455 mg/mL, respectively. CsA had the highest solubility in MYS-40 and, therefore, it was the best of the three surfactants for preparing clear solutions. Accordingly, MYS-40 was used throughout this study to prepare aqueous CsA solutions. The relationship between MYS-40 concentration and CsA solubility at 25, 40 and 60 °C is shown in Figure 1. It indicates that at each of these temperatures CsA solubility increased linearly. A 2 % MYS-40 aqueous solution was chosen to ensure that 0.1 %CsA was completely soluble in aqueous solution.

Ocular Distribution

Ocular distribution resulting from exposure to 0.1 % Aq-CsA was compared to those obtained with Oil-CsA and Em-CsA. The results in Table Ia and Ib show that most of the CsA administered in an Aq-CsA formulation distributed to ocular surface tissues such as the corneal epithelium, corneal stroma/endothelium, and bulbar conjunctiva. A small amount of CsA distributed to the lacrimal gland, the anterior and posterior segments. A trace amount of CsA was detected in the blood at all time points.

DISCUSSION

This study was undertaken to identify a CsA containing ophthalmic formulation that may be more desirable for de-

Table Ia. CsA Levels (ng-eq/g) in the Ocular Tissues after Single Instillation (50 μL) of Three CsA Formulations in the Rabbits

	Aq-CsA time (h)			Em-CsA time (h)		
Ocular tissue	1	4	24	1	4	24
Lacrimal gland	6.38 ± 8.09	3.06 ± 0.74	1.00 ± 0.42*	1.50 ± 1.46	1.21 ± 0.33	0.51 ± 0.13**
Harder gland	7.28 ± 10.86	1.70 ± 0.05	0.42 ± 0.15**	0.61 ± 0.20	0.59 ± 0.17	0.27 ± 0.07**
Bulbar conjunctiva	1373.80 ± 548.31**	542.73 ± 80.17**	27.83 ± 7.06	446.04 ± 164.73	287.60 ± 138.99*	19.70 ± 6.18*
Corneal epithelium	20234.65 ± 6348.76**	12247.78 ± 4775.28**	4932.76 ± 2143.96**	4952.95 ± 1348.05	5317.78 ± 1723.18	2662.46 ± 653.42
Corneal Stroma–endothelium	601.74 ± 485.45*	595.43 ± 230.85**	491.32 ± 114.00**	82.07 ± 38.14	175.45 ± 62.73	201.64 ± 69.33*
Aqueous humor	3.48 ± 1.35	1.22 ± 0.39**	1.45 ± 0.31	2.66 ± 0.62	0.86 ± 0.38	0.52 ± 0.15
Choroid–retina	2.28 ± 1.26	1.80 ± 0.48	1.54 ± 0.95	0.87 ± 0.34	0.85 ± 0.27	0.36 ± 0.08
Iris–ciliary body	3.56 ± 1.31**	5.44 ± 2.49**	23.95 ± 8.47**	1.76 ± 0.42	1.61 ± 0.58	10.07 ± 2.74
Whole blood	1.02 ± 0.64	0.31 ± 0.13	ND[b]	0.30 ± 0.06	0.21 ± 0.09	ND[b]
Blood plasma	0.29 ± 0.17	0.31 ± 0.21	ND[b]	—[a]	—[a]	—[a]

[a] Not assayed.
[b] ND: less than detection limit.
Each value represents mean ± SD of four experiments.
* $P < 0.05$; ** $P < 0.01$ vs. castor oil (Dunnett multiple comparison test).

Table Ib. CsA Levels (ng-eq/g) in the Ocular Tissues after Single Instillation (50 μL) of Three CsA Formulations in the Rabbits

Ocular tissue	Oil-CsA time (h) 1	4	24
Lacrimal gland	5.74 ± 7.94	5.22 ± 5.86	2.17 ± 0.99
Harder gland	4.56 ± 5.08	2.33 ± 2.17	1.64 ± 0.30
Bulbar conjunctiva	104.29 ± 30.53	88.46 ± 44.11	59.07 ± 33.31
Corneal epithelium	687.89 ± 427.28	744.26 ± 148.20	643.94 ± 150.51
Corneal stroma–endothelial	10.00 ± 4.20	10.51 ± 3.05	31.33 ± 7.02
Aqueous humor	2.73 ± 0.61	0.28 ± 0.05	ND[a]
Choroid–retina	2.38 ± 1.72	1.57 ± 0.92	0.92 ± 0.57
Iris–ciliary body	1.24 ± 0.34	0.38 ± 0.00	1.16 ± 0.27
Whole blood	ND[a]	0.19 ± 0.03	ND[a]
Blood plasma	ND[a]	0.14 ± 0.04	ND[a]

[a] ND; less than detection limit.
Each value represents mean ± SD of four experiments.

livering larger amounts of this immunosuppressant into ocular tissues. Such a route of application would be preferable because it substantially reduces the likelihood of adverse effects that result from CsA systemic administration. Prior to this study, it was not possible to envisage the use of an aqueous formulation that also had no irritating ocular side effects. The only formulations that could be used to dissolve CsA required an oil base. However, these formulations (i.e., with up to 1% CsA) did not result in much ocular tissue CsA accumulation. Furthermore, an oily formulation for ophthalmic use is undesirable because it causes vision blurring and is irritating. We have shown that the level of CsA accumulation by most ocular tissues can be markedly enhanced by applying an aqueous CsA formulation that contains the nonionic surfactant MYS-40. High CsA concentrations were observed in the corneal stroma–endothelium at all time points after a single topical instillation of the newly formulated aqueous solution (Aq-CsA) in comparison with those for Oil-CsA and Em-CsA. At 1-h post instillation, CsA levels for Aq-CsA were 60.2 and 7.3 times higher than that for Oil-CsA and Em-CsA, respectively (Table I). This pattern was also observed in the bulbar conjunctiva. CsA exhibited the following penetration order: Aq-CsA > Em-CsA > Oil-CsA.

The area under the curve (AUC_{0-12}) and AUC ratio, which were calculated using AUC (Oil-CsA) as a control, are shown in Table II. The AUC for Aq-CsA in the corneal stroma-endothelium, which is the target tissue for preventing corneal allograft rejection after corneal transplantation surgery, was 28.5 and 3.1 times higher than those for Oil-CsA and Em-CsA, respectively.

Table II. Comparison of AUC_{0-12} for the Corneal Stroma–Endothelium, Bulbar Conjunctiva, and Lacrimal Gland after Single Instillation of 0.1 % Cyclosporine A in Three Different Formulations in the Rabbits

Ocular tissue	Oil-CsA AUC	Oil-CsA Ratio	Aq-CsA AUC	Aq-CsA Ratio	Em-CsA AUC	Em-CsA Ratio
Corneal stroma–endothelium	454.2	1	12962.1	28.5	4198.2	9.2
Bulbar conjunctiva	1816.6	1	9267.3	5.1	4396.5	2.4
Lacrimal gland	93.2	1	58.0	0.6	22.0	0.2

AUC_{0-12}: ng-eq · h/g. Each ratio represents AUC (each formulation)/AUC (Oil-CsA).

It appears that CsA penetration into the ocular surface tissues is affected by the release rate of CsA from its carrier in the dispersion medium. In an aqueous medium containing a nonionic surfactant, micelles are formed and CsA was dissolved in them. The size of micelles is much smaller than that of emulsion droplets, which is 200 nm in mean diameter (7). Accordingly, much more CsA can be released from micelles for drug penetration because they have a much larger surface area, enabling them to bind much more drug. Another impediment limiting drug penetration is that CsA has a strong affinity for lipophilic vehicles, such as vegetable oils, because their partition coefficient is 6. Therefore, CsA release is poor from oily vehicles and o/w emulsion. Perry *et al.* determined the cornea CsA levels in patients, who were topically treated with 0.5 % CsA every 15 min for one hour before corneal surgery (8). The mean cornea CsA level was 3679 ng/g in 9 patients. In our study, the maximal CsA corneal level reached was 2112 ng/g (total cornea) at 1 h after only a single administration of 0.1 % aqueous CsA solution. This value is equal to the sum of the normalized CsA content on a wet weight basis in the corneal epithelial, stromal and endothelial layers. This result suggests that the CsA concentration in the ophthalmic solution could be reduced below 0.1 % to achieve the same extent of penetration as in patients. Alternatively, even greater penetration may be possible by using the 0.1 % aqueous CsA solution.

CONCLUSIONS

A cyclosporine aqueous ophthalmic solution that uses polyoxyl 40 stearate as a solubilizer for CsA has been designed. An ocular pharmacokinetic study using ^{3}H-CsA showed that the distribution of CsA in ocular tissues, such as cornea, bulbar conjunctiva, and lacrimal gland, after topical instillation of the aqueous formulation was superior to that after instillation of an o/w emulsion and an oily formulation. We suggest that this aqueous CsA ophthalmic formulation will be clinically useful in the treatment of immune-mediated ophthalmic diseases.

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MATERIAL SAFETY DATA SHEET

LAMBENT TECHNOLOGIES CORP.
3938 Poratt Drive
Gurnee, IL 60031
(800) 432-7187

CHEMTREC EMERGENCY RESPONSE
TOLL FREE NUMBER: (800) 424-9300
INTERNATIONAL NUMBER : (703) 527-3887

1. PRODUCT IDENTIFICATION

Product Name: **LUMULSE™ POE-40 MS KP**
Synonym: Polyethylene glycol monostearate

2. COMPOSITION / INFORMATION ON INGREDIENTS

	CAS Number	Weight %	ACGIH TLV	OSHA PEL
Ethoxylated stearic acid	9004-99-3		Not est.	Not est.

3. HAZARDS IDENTIFICATION

Potential Health Effects

INHALATION: No evidence of adverse effects from available information.

EYE CONTACT: May cause irritation. Irrigate eye with water for at least 15 to 20 minutes. Seek medical attention if symptoms persist.

SKIN CONTACT: No evidence of adverse effects from available information.

INGESTION: No evidence of adverse effect from available information.

4. FIRST AID MEASURES

EYES: Irrigate eyes with a heavy stream of water for at least 15 to 20 minutes.

SKIN: Wash exposed areas of the body with soap and water.

INHALATION: Remove from area of exposure, seek medical attention if symptoms persist.

INGESTION: Give one or two glasses of water to drink. If gastro-intestinal symptoms develop, consult medical personnel. (Never give anything by mouth to an unconscious person.)

5. FIRE FIGHTING MEASURES

FLASH POINT (Method Used): > 350°F (COC)
FLAMMABILITY LIMITS: None known

EXTINGUISHING MEDIA: Dry chemical, foam, halon, CO_2, water spray (fog). Water stream may splash burning liquid and spread fire.

SPECIAL FIRE FIGHTING PROCEDURES: Use water spray to cool drums exposed to fire.

UNUSUAL FIRE AND EXPLOSION HAZARDS: Firefighters should use self-contained breathing apparatus to avoid exposure to smoke and vapor.

6. ACCIDENTAL RELEASE MEASURES

SPILL CLEAN-UP PROCEDURES: Remove sources of ignition, contain spill to smallest area possible. Stop leak if possible. Pick up small spills with absorbent materials such as paper towels, "Oil Dry", sand or dirt. Recover large spills for salvage or disposal. Wash hard surfaces with safety solvent or detergent to remove remaining oil film. Greasy nature will result in a slippery surface.

7. HANDLING AND STORAGE

Store in closed containers between 50°F and 120°F. Keep away from oxidizing agents, excessive heat, and ignition sources. Store and use in well ventilated areas. Do not store or use near heat, spark, or flame; store out of sun. Do not puncture, drag, or slide this container. Drum is not a pressure vessel; never use pressure to empty.

8. EXPOSURE CONTROLS / PERSONAL PROTECTION

RESPIRATORY PROTECTION: If vapors or mists are generated, wear a NIOSH approved organic vapor/mist respirator.

PROTECTIVE CLOTHING: Safety glasses, goggles, or face shield recommended to protect eyes from mists or splashing. PVC coated gloves recommended to prevent skin contact.

OTHER PROTECTIVE MEASURES: Employees must practice good personal hygiene, washing exposed areas of skin several times daily and laundering contaminated clothing before re-use.

9. PHYSICAL AND CHEMICAL PROPERTIES

Boiling Point, 760mm Hg:	> 200°C
Specific Gravity, (H_2O=1):	N/A
Vapor Pressure, mm Hg:	< 1
Vapor Density, (Air=1):	> 1
Volatiles, % by Volume:	< 1% (water)
Evaporation Rate, (Butyl Acetate=1):	< 1
Solubility in Water, % by Volume:	Soluble
Appearance and Odor:	White waxy flake with a bland odor

10. STABILITY AND REACTIVITY

GENERAL: This product is stable and hazardous polymerization will not occur.

INCOMPATIBLE MATERIALS AND CONDITIONS TO AVOID: Strong oxidizing agents

HAZARDOUS DECOMPOSITION PRODUCTS: Combustion produces carbon dioxide, carbon monoxide along with thick smoke.

11. DISPOSAL CONSIDERATIONS

Waste may be disposed of by a licensed waste disposal company. Contaminated absorbent material may be disposed of in an approved land fill. Follow local, state and federal disposal regulations.

12. TRANSPORT INFORMATION

UN HAZARD CLASS: N/A

13. REGULATORY INFORMATION

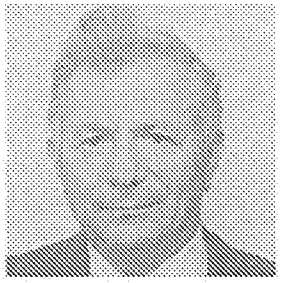
OSHA STATUS: This product is not hazardous under the criteria of the Federal OSHA hazard Communication Standard 29 CFR 1910.1200. However, thermal processing and decomposition fumes from this product may be hazardous as noted in Sections 3.
TSCA STATUS: The components of this product are listed on TSCA.

14. OTHER INFORMATION:

NFPA Codes: Health: 1 Fire: 1 Reactivity: 0

Revision Notes: 6/1/99 Change brand name from Lexal to LUMULSE®
4/22/02 Change International emergency number
10/9/02 Add KP designation.
8/22/03 Change company contact information and emergency contact information.

This information relates only to the specific material designated and may not be valid for such material used in combination with any other materials or in any other process. Such information is to the best of the company's knowledge and believed accurate and reliable as of the date indicated. However, no representation, warranty or guarantee of any kind, express or implied, is made as to its accuracy, reliability or completeness and we assume no responsibility for any loss, damage or expense, direct or consequential, arising out of use. It is the user's responsibility to satisfy himself as to the suitableness and completeness of such information for his own particular use.



Our Experiences in Processing Maize (Corn) Germ Oil

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ABSTRACT

This paper discusses the properties and composition of corn oil. Influences of processing steps on oil quality are described, along with alkali and physical refining, degumming, bleaching, dewaxing, deacidification-deodorization. A new method for wet degumming-bleaching and deacidification-deodorization is presented. Flowsheets and other data are given for a new 150 metric tons per day of corn oil plant.

Corn oil is produced as a byproduct of two of the corn-using industries: the starch industry, in which maize germ is obtained in the wet degermination process (the oil content of the germ obtained in this process is ca. 50%); and the corn meal industry which produces hominy, grits, corn flakes, etc. Here the germ is obtained in the dry degermination process and contains from 10% to 24% oil.

Corn oil is a minor commodity in the oil market. The increase in use of corn oil as salad and cooking oil, and in margarine, is due to the awareness of the importance of polyunsaturated fatty acids in the diet, its tocopherol content and oxidation stability. Wide publicity has been given to the nutritional properties of corn oil as a good source of essential fatty acids. Corn oil production has increased markedly due to the increased consumption of high fructose corn syrup produced from the corn starch.

Corn oil composition depends on the seed type and climatic conditions. It is known that oil obtained from maize grown in the north has a higher iodine value (IV) than the same type grown farther south.

The average analysis of crude maize oil processed in South African factories built by H.L.S. Ltd. is 3-6% free fatty acids (FFA), iodine value of 110-125, and 300-1000 ppm phosphorus.

Oil from different corn, can be expected to have different compositions. There is a direct relationship between the iodine value of corn oil and its linoleic acid content. The equation is Y (%C18) = 0.95 (IV) - 61.8. Regarding the phospholipids of corn oil: more than 50% of them contained inositol, the rest being glyceryliphosphatidylcholine, phytoglycolipid, etc.

Corn oil contains traces of waxes, which are esters of myricil alcohol with tetracosanoic acid. According to literature, ceryl alcohol esters were also identified. The melting point of these waxes is 81-82 C.

An important component of the unsaponifiable fraction of corn oil is tocopherol (ca. 0.1%), mainly γ-tocopherol which has antioxidant potential activity. The main pigments in crude corn oil are xanthophylls and carotenes.

TABLE I

Fatty Acid Composition of Maize Oil

Fatty acid	Codex Alimentarius (tentatively adopted) (%)	South Africa (%)	USA (%)
C12 (Lauric)	0.1	0.4	0.1
C14 (Myristic)	1.0	0.2	0.2
C16 (Palmitic)	8.0-19	11.5	11.8
C16 (Palmitoleic)	0.5	0.1	
C18 (Stearic)	0.5-4	2.0	2.0
C18 (Oleic)	19.0-48	38.7	24.3
C18 (Linoleic)	34.0-62	44.3	61.9
C18 (Linolenic)	2.0	1.1	0.7
C20 (Arachidic)	1.0	0.6	1.7
C22 (Behenic)	0.5	0.1	
C22 (Erucic)	0.5	0.3	
C24 (Lignoceric)	0.5	0.3	

TECHNOLOGICAL PROCESSING

The method used to obtain the maize oil, the history of the grain, the storage and handling of the germ—all have an important influence on the quality of the crude oil. In our experience, wet milled germ must be processed by prepressing extraction. Some large starch factories also have final presses which give cakes with 6% oil.

The germ obtained by dry degermination can be direct-extracted in solvent extraction plants. Preparation of germ with a small percentage of fines consists of the usual cracking, conditioning and flaking. For separated dry germ with a high percentage of fines (as in South Africa), it is necessary to pellet the germ before extraction.

For the direct solvent extraction of the germ, in the form of pellets or flakes, we developed the TOM extractor which has been described at previous AOCS meetings so we will point out only its main advantages.

In the TOM extractor, we turn over the material at its half-way point inside the extractor (Fig. 1). This inversion mixes the material and destroys the impermeable layer which the fines form at the top of the material bed. The starch content of the germ is not sufficiently gelatinized and this makes the extraction more difficult. For this reason we try to work with a shallow bed of material in the basket. The starch also absorbs more hexane than normal oilseed flakes. In the TOM extractor, we can work in the beginning by immersion and in the second stage of extraction, continue with percolation. We have a large number of extracting stages and good separation of the various miscella concentrations. To obtain a clean miscella, a multiple system of hydrocliones is introduced for materials with a high percentage of fines.

Several years, in cooperation with an oil plant in Durban, South Africa, belonging to the Tiger Oats Group, we developed and supplied a physical refining plant to deacidify maize oil with a high fatty content (average 23.6%).

After normal wet degumming, the oil was heated and during continuous mixing, 0.3-0.5% phosphoric acid (conc. 85%) was added. At 90 C, 2% bleaching earth was introduced and the temperature brought to 110 C under vacuum for 20-25 min. After cooling and filtration, the oil was introduced into the deacidifier-deodorizer with ca. 5% of direct steam. Over 220 C, the fatty acids started to distill. The final temperature of distillation was 250 C when

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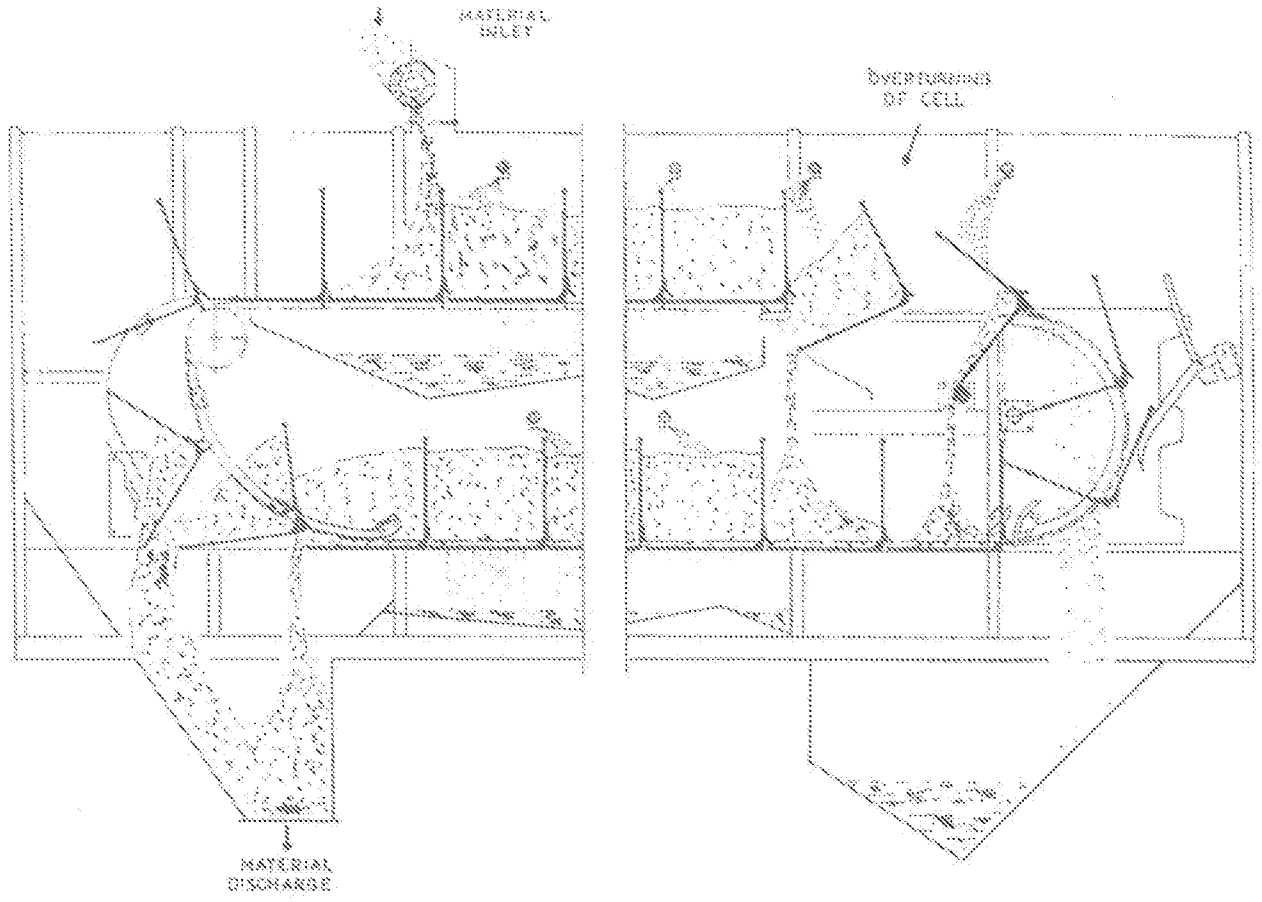

FIG. 1. TOM extractor.

deodorization started. Using this system, the FFA could be decreased to 0.3-1%.

After this operation, it was necessary to postrefine the oil. It was very difficult to neutralize this oil which had a high monoglyceride content. We continued our research and in the last 2-3 years, developed a new, patented method of special wet degumming (SWD) which eliminated the need to postrefine and directly gives deacidified-deodorized oil.

The main advantage of the SWD system is reduced phosphorus and metal content of the oil. The result is that in the bleaching process the amount of bleaching earth required is no larger than the amount usually needed for bleaching after neutralization. In this case, the yield of refined oil is better and there are no extra expenses for bleaching earth. Also, the more complete elimination of phosphorus and metals makes the stability of the oil similar or better than with classical refining methods.

The success of this new method is attributable to several factors: a reactive food-grade acid, the proper temperature of reactions, optimum retention time and an efficient, inexpensive flocculating agent.

H.L.S. is now building, together with Epic Oils, a 150 ton/24 hr plant for maize oil. The special wet degumming (SWD) and bleaching system for this plant is shown in Figure 2.

This process begins with pumping the oil into the system through a heat exchanger, which brings it to the proper temperature. Other exchangers along the way maintain the temperature of the oil at the correct levels. In the first mixer, the oil is treated with a food-grade acid proportioned by a dosing pump. Efficient agitation insures total contact between the acid and the oil. When the action is completed, the oil is pumped to the hydration mixer where a second dosing pump introduces the proper amount of aqueous flocculant solution. After extensive mixing, the treated oil flows into a holding tank where slower precipitation is completed and the flocculs grow large and heavy.

The next step is the centrifuge where the solids are separated. The precipitated gums can be dried and sold to manufacturers of commercial lecithin.

The degummed oil goes to the drier where all water is removed. Part of the oil enters a mixing tank where a predetermined quantity of bleaching clay is added. The slurry and the degummed oil are pumped to the continuous bleacher and agitated under vacuum for about half an hour before filtering. A pair of vertical filters separates the bleaching earth from the oil—one is in operation while the other is being cleaned.

The filtered oil is cooled, and passed through a safety filter to remove any remaining traces of clay. It must now be deacidified and deodorized. Although there are many ways and systems to accomplish this final step, the H.L.S. continuous deacidification and deodorization system (CDD) has special and unique advantages (Fig. 3) the main

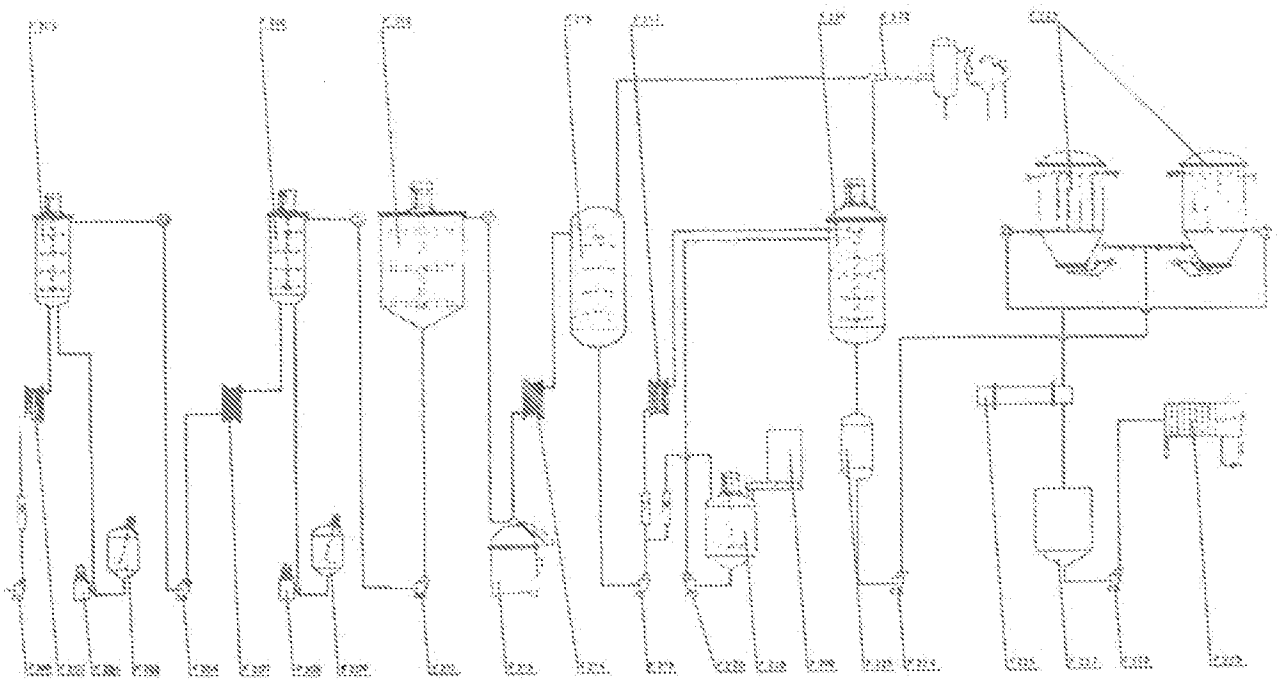

FIG. 2. Special wet degumming (SWG) and continuous bleaching.

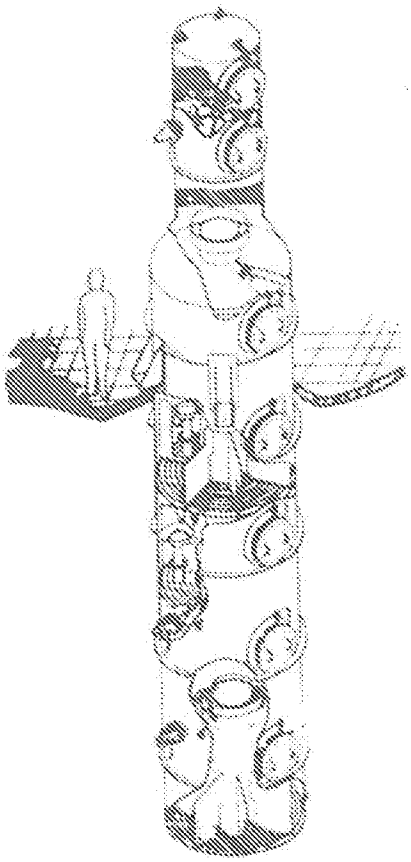

FIG. 3. Continuous deacidifier/deodorizer.

one being steam and energy economy, as a result of very efficient heat exchanger which cools the final product as it heats the incoming oil, and a low amount of stripping steam accomplished by highly effective steam dispersion through a large number of nozzles, with a low oil level preventing coalescence of the steam bubbles.

These two factors bring the total steam consumption to 100-120 kg/ton of oil (with cooling water temperature of 20-25 C). Other features of the H.L.S. deacidifier-deodorizer are continuous and semicontinuous operation in the same unit because of the special timing valves which allow working semicontinuously, or overflow pipes which allow the bypassing of the closed valves. This feature enables rapid changes of oil stocks.

We have introduced dividing walls which enable the system to operate in the continuous way on the principle of "first in, first out".

To illustrate use of the newly developed physical refining system, results of processing in our pilot plants of maize oils from several sources are shown in Tables II and III.

These examples show that with this new method we obtain good oils (with better yields also) for maize oils with high acidity. From oil with 8.4-14% FFA corresponding to a Wesson loss of 16-16%, under the best conditions, the results after classical (caustic) refining are 85-75% refined oil. The same oil, after steam refining, gives us 89-80% refined oil.

Other advantages of physical refining: no ecological problems as with soapstock splitting; better quality of fatty acids; and lower initial investment in equipment--there is no need of neutralizing and washing centrifuges, no need for splitting section. Only one centrifuge is required for the separation of the gums.

DEWAXING AND WINTERIZATION

Maize oil has small amounts of waxes which solidify at low

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TABLE II

Maize (Corn) Oil from Dry Degermination—South Africa

Sample:	D	M	B	E
FFA (%)	8.7	14	8.4	18
P (ppm)	50	319	391	323
Lovibond (1")	Y=70, R=7.3	Y=35, R=4.6	Y=70, R=7.4	Y=50, R=4.7
After SWD and bleaching (2%)				
P (ppm)	0.9	7.6	2.9	2.35
After deacidification-deodorization				
FFA (%)	0.05	0.02	0.07	0.04
PV (meq/kg)	0	0	0	0
Lovibond (1")	Y=20, R=2.7	Y=15, R=1.9	Y=20, R=2	Y=30, R=2.2

TABLE III

Maize (Corn) Oil from Wet Degermination

Source:	Israel	Israel	Belgium	South Africa
From:	Press	Extraction	Extraction	Extraction
FFA (%)	4.4	4.8	1.79	2.1
P (ppm)	185	180	640	285
Lovibond (1")	Y=40, R=5.5	Y=40, R=9	Y=45, R=10.5	Y=40, R=5
After SWD and bleaching (2%)				
P (ppm)	4.8	5.2	2	2.85
After deacidification-deodorization				
FFA (%)	0.03	0.04	0.02	0.03
PV (meq/kg)	0.1	0	0	0
Lovibond (5¼")	Y=30, R=4.1[a]	Y=14, R=0.9	Y=6, R=0.9	Y=15, R=1

[a]Color fixation by excessive temperature in cookers.

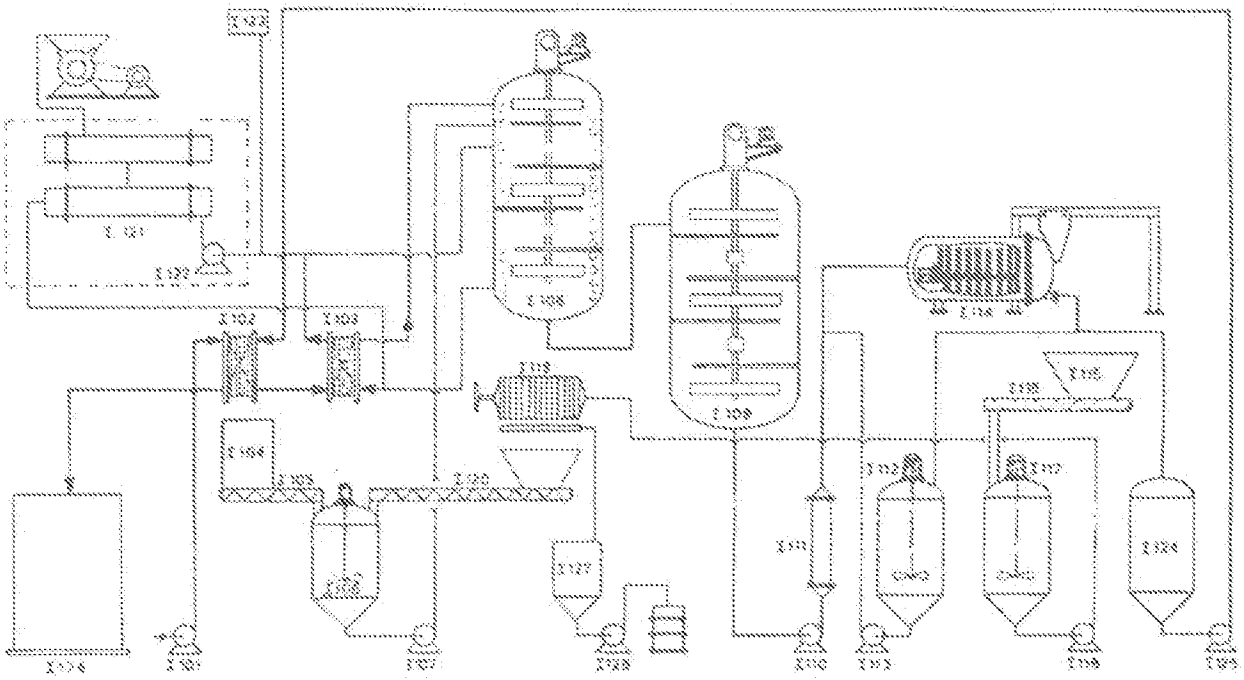

FIG. 4. Continuous winterizing.

temperatures and cause the oil to be turbid. Recent research on the triglyceride structure of corn oil showed that no triglyceride fraction that consisted wholly of saturated fatty acids was obtained and only a small percentage of disaturated triglycerides was found. As average, the triglyceride structure of corn oil can be represented as: S3 = 0, S2U = 2.2, SU2 = 40.3, U3 = 57.5.

Although the amount of disaturated triglycerides is quite small, it also contributes (in addition to the waxes) to the turbidity of the oil at low temperatures, and therefore has to be eliminated as well.

The dewaxing and winterizing of maize oil is difficult. In the new plant for Epic, we have installed our winterization system which is shown in Figure 4. The main conditions for winterization are: clean, degummed and dry oil—for this reason we place the winterizing after bleaching and before deacidification; optimum oil cooling temperature and low temperature difference by cooling to permit formation of filterable crystals; maturation after crystallization before filtration; and a suitable filter—we prefer the horizontal tank filters.

DESCRIPTION OF PROCESS

The bleached oil is precooled in a plate-type heat exchanger using the cold filtered winterized oil as the cooling media. Then it is further cooled in a plate type cooler using glycol solution as chilling agent. The chilled oil is introduced into the continuous crystallizer where it is cooled at a controlled rate to the optimum crystallization temperature while being gently agitated. A certain amount of filter aid (Kiselgur) is added to the crystallizer in the form of slurry which was prepared in a special mixing tank. This powder serves as crystallization nuclei and facilitates the oil filtration. After passing the crystallizer, the oil is introduced into the maturator in which the crystals get their final form suitable for filtration. Before filtration, the oil is heated somewhat to accelerate filtration rate.

The filter aid can be recuperated and reused. For this an additional filter is provided in which the powder is separated from the waxes and the stearines. After this separation, the waxes and stearines can be used in the margarine industry. In our pilot plants, we have also obtained good winterization of maize oil by crystallizing the oil with a wetting agent together with magnesium sulfate.

Although corn is one of the principal crops in the world, only a small part of it is used for producing maize oil. We notice an increase in the production of maize oil, but it is still not significant. The developing of alcohol plants for fuel from corn could contribute to the expansion of degerminating and oil production. Today the technology for processing maize germ and oil is sufficiently advanced to be able to handle the increased production of this premium oil.

Effect of a cyclosporine A delivery system in corneal transplantation

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Keywords: *cyclosporine* · *corneal allografts* · *polymer* · *immunosuppression*

Objective To test the immunosuppressive effect of cyclosporine (Cs) in a polymer placed in the anterior chamber of corneal allograft recipients.

Methods Wistar inbred rats with vascularized corneas were recipients of corneal allografts from Sprague-Dawley donor rats. Rats underwent penetrating keratoplasty and were divided randomly into four groups: untreated control animals (UCA); Cs-polymer anterior chamber recipients (CPA); co-polymer subconjunctival recipients (CPS); and Cs-olive oil drop recipients (COO). Grafts were examined by slit lamp every 3 days and clinical conditions were scored. Cs concentration in the aqueous humor was assayed at 1, 2, and 4 weeks. At 1, 2 and 4 weeks after transplantation, the operated eyes were collected for histopathological evaluation of the grafts.

Results The median survival time of the allografts was 8.2±1.48 days for the UCA group, 11.4±2.50 days for the CPS group, and 17.0±2.00 days for the CPA group. There was a statistically significant difference ($P<0.05$) between survival time of the allografts in the animals of the CPA group compared to the other groups of graft recipients. Significantly higher concentrations of Cs were found in the eyes given an anterior chamber implant of Cs-polymer, compared to other treatment groups or untreated rats. A transient inflammatory response in the anterior chamber was observed in the CPA group.

Conclusions Cs-polymer placed in the anterior chamber significantly prolongs corneal allograft survival time in a high risk corneal graft rejection model. This intraocular delivery system may be a valuable adjunct for the suppression of immune graft rejection.

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Corneal transplantation is one of the most common allografts performed. While 90% of these allografts survive and function satisfactorily, immune graft rejection is a major cause of graft failure. In "high risk" patients, 60% of corneal allografts can be rejected in spite of the use of topical steroids.

Cyclosporine (Cs) has been found to be an effective immunosuppressive agent and is widely used in organ transplantation in humans.[1] Systemic administration of Cs has been found to be effective in prolonging corneal allograft survival in experimental animals and in patients. However, the systemic side effects of Cs include nephrotoxicity and hypertension, which mitigate against the use of this drug in corneal allografting. Consequently, there is considerable interest in developing a delivery system which could achieve therapeutically effective drug concentrations localized to the anterior segment.

It has been reported that topical administration of Cs prolongs corneal allograft survival in both rabbits and rats.[2] The problem of drug insolubility and failure to achieve clinically significant drug concentrations in the cornea and anterior chamber are, however, a limitation of this approach.

We developed a Cs-polylactioglycollic acid polymer implant and tested the implant in the anterior chamber of rats with corneal allografts.

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METHODS

Animals

Wistar inbred male rats, weighing 250 g each, and Sprague-Dawley (SD) rats of mixed sexes, weighing 250 g each, were obtained from the animal care center at Shandong Medical University, China. The Wistar rats served as recipients and the SD rats were used as corneal allograft donors. The animals were maintained and handled according to the ARVO Resolution on the Use of Animals in Research.

Drug delivery system

Cs powder (Novartis, Ltd., Basel, Switzerland) was formulated into the polymer, polylactioglycollic acid (PLGA), by the Institute of Chemistry, the Chinese Academy of Sciences. The polymer was shaped into cylinders 1.5 mm long and 2 mm in diameter and the ratio of Cs to PLGA was 6:4. Each cylinder contained 0.3 mg of Cs.

Penetrating keratoplasty

Three loops of 8-0 silk suture were placed in the anterior cornea of one eye of Wistar rats and vascularization was induced in 10 – 12 days. When the blood vessels had grown into the recipient cornea, the sutures were removed and a donor cornea from SD rats was transplanted into the site.

There were 10 grafted animals in each of the four treatment groups. In the CPA group, the recipient eye was implanted with Cs-polymer in the anterior chamber immediately before closure of the surgical wound. In the CPS group, the grafted eye received a Cs-polymer implant in the subconjunctiva. In the COO group, the grafted eye received 1% Cs drops three times a day for four weeks following surgery. In the UCA group, the grafted eye was not treated throughout the observation period.

Another nine Wistar rats which did not undergo penetrating keratoplasty were anesthetized and given implants of the Cs-polymer in the anterior chamber. These rats served as control animals for the observation of toxicity related to the Cs-polymer implant.

Clinical evaluation of the grafts

Recipients of corneal allografts were examined every 3 days for four weeks by a blinded observer using a slit lamp microscope. Clinical appearance of each graft was scored using the following three criteria: graft opacity, graft edema, and graft vascularization. The scoring scheme was based on previously described grading systems.[3] Briefly, the scoring scale ranged from 0 to 4. Grade 0 is a clear graft. Grade 1 is a slightly hazy graft that is moderately thickened and has a mild cellular infiltrate. Grade 2 is a distinctly hazy graft which is thickened. Grade 3 is a hazy graft which is vascularized over at least one-half of its diameter. Grade 4 is a completely opaque graft that is vascularized over 75% of its diameter. Grafts with grade 3 or higher were recorded as the rejected.

Assay of cyclosporine in the aqueous humor

At 1, 2 and 4 weeks, rats were anesthetized and 25 μl of aqueous humor was obtained, diluted 1:4, and analyzed for Cs concentration with a fluorescent polarization immunoassay.

Histopathology

Rats were sacrificed at 1, 2, and 4 weeks after surgery. Eyes were fixed in 10% neutral buffered formalin, processed for paraffin embedding and sectioning, and 8 micron sections were stained with hematoxylin and eosin. Eyes were evaluated by an ocular pathologist. Cells and flare in the anterior chamber, inflammatory cells and disruption of the normal cellular structure in the cornea and the iris were graded.

Data analysis

Graft survival time was compared using the two-tailed Student's t test, Q test and mean square deviation analysis.

RESULTS

Effect of cyclosporine on corneal allograft survival

Nine animals were excluded from the study due to chronic inflammation of the graft, cataract development, and failure to reform the anterior chamber after surgery.

In all four groups, mild corneal edema was seen immediately after surgery. The edema disappeared in three days after transplantation. Enlarged blood vessels were observed at the graft-host junction, particularly around the sutures. By days 5 – 7 after surgery, some of these vessels began to enter the grafts.

All ten grafts in the UCA group were rejected within 10 days. The median survival time of these grafts was 8.2 ± 1.48 days. In the CPA group, grafts were rejected between 15 and 23 days with a median survival time of 17 days. Animals in the CPS group rejected the corneal allografts in a median time of 11.4 days and none of these grafts survived beyond 13 days. In the COO group, the mean survival time of the grafts was 10.6 days and no grafts survived beyond 13 days (Table 1).

Statistically, there was no significant difference in the mean survival time of the grafts between the UCA and COO or CPS groups. Q test analysis revealed that $P > 0.05$. There was a

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statistically significant difference in the median survival time between the CPA group and the other three groups of animals ($P < 0.001$).

Cyclosporine concentration in the aqueous humor

Cs was not found in the aqueous humor in the UCA group. In the COO group the aqueous concentration was 47.9 ng/ml at one week after surgery. In the CPS group, the aqueous humor levels at one week were 56.5 ng/ml. The difference between the COO and CPS groups was statistically significant ($P < 0.05$). The concentration of Cs in the CPA group at one week was 121.7 ng/ml. This concentration of Cs was significantly higher than the above two groups ($P < 0.005$) (Table 2).

Table 1. Effect of cyclosporine on corneal allograft survival

Treatment groups	Number of grafts	Rejection (%)	Median survival time (days)
Untreated allografts (UCA)	10	100	8.2 ± 1.48
1% Cs drops (COO)	10	100	10.6 ± 1.90
Cs-polymer (subconjunctival) (CPS)	10	100	11.4 ± 2.50
Cs-polymer (anterior chamber) (CPA)	10	100	17.0 ± 2.00

Allografts were examined every other day and a clinical score assigned. When a graft reached grade 3 (hazy and vascularized over one-half its diameter) it was considered rejected.

Table 2. Cyclosporine in the aqueous humor

Treatment groups	Number of eyes sampled	Cs concentration (ng/ml)
Untreated (UCA)	10	0
1% Cs drops (COO)	5	47.9 ± 3.48
Cs-polymer (subconjunctival) (CPS)	10	56.5 ± 6.25
Cs-polymer (anterior chamber) (CPA)	10	121.7 ± 16.79

The aqueous humor concentration of cyclosporine was determined using a fluorescent polarization assay. The data are given as the mean drug concentration plus/minus the standard deviation of the mean.

A time course of Cs concentration in the aqueous humor of the CPA group revealed that the highest drug concentration was present one week after implantation. The concentration fell slightly by the end of two weeks and by four weeks, drug levels had declined significantly (Table 3).

Table 3. Delivery of cyclosporine by anterior chamber polymer implant

Time after implantation (wk)	Number of eyes sampled	Cs concentration (ng/ml)
1	5	133.10 ± 18.30
2	5	124.56 ± 9.65
4	5	107.45 ± 11.48

Eyes receiving cyclosporine-polymer implants were tested for drug concentration at the times indicated. The data are given as the mean drug concentration plus/minus the standard deviation of the mean.

Histopathology in CPA group

At fourteen days after implantation of the Cs-polymer, mild inflammation and edema at the graft-host wound margin was observed. The inflammatory infiltrating cells consisted of leukocytes and a few macrophages and lymphocytes. The inflammatory infiltrates regressed by 28 days after implantation and the corneas remained clear. The corneal endothelium appeared normal throughout the observation period. The capsule of the lens was intact, and retinal changes in these eyes were observed.

DISCUSSION

In high risk circumstances, the immunologic privilege of tissues may be altered. There are macrophages in the iris adjacent to the anterior chamber and these cells and corneal Langerhans cells can serve as antigen presenting cells.[4] In this circumstance, it may be especially important to achieve and sustain high levels of Cs in the anterior chamber in order to prevent graft rejection in a high risk situation. In this study, implantation of a polymer containing Cs into the anterior chamber significantly prolonged the survival of corneal allografts. We suggest that Cs of the polymer implant in the anterior chamber suppresses the response of T lymphocytes to antigen-presenting cells and aborts the initial recognition of graft antigens. Transient inflammation and corneal edema were observed.

The PLGA delivery system used here has been found to be nontoxic and biodegradable. The polymer dissolves following implantation into humans and has been widely used to promote bone healing after surgery.[5] In this study there was no evidence of toxicity related to the Cs incorporated into the PLGA polymer. Aside from transient inflammation noted four days after implantation, the eyes appeared normal. We suggest that this anterior chamber delivery system may be a valuable approach to the delivery of Cs.

Studies on the injection of Cs into the vitreal cavity of rabbits found it to be nontoxic at this site.[6,7]

The aqueous humor concentration of Cs required to prevent immune graft rejection is not known. Furthermore, it is not clear how long the drug must be sustained in order to protect the corneal allograft. In this study, corneal allograft rejection occurred when Cs levels in the aqueous humor fell below 110 ng/ml. Future studies are needed to determine the aqueous humor levels of Cs required to prevent graft rejection in patients. Pearson et al[8] studied a delivery system providing long-term release of Cs in the vitreous. Concentrations as high as 500 ng/ml were recorded. This is significantly higher than 100 ng/ml found in the vitreous of patients with ocular Bechet's disease and pan uveitis who were treated with 5 mg/kg/d systemically.[9]

Lymphokine production by activated T lymphocytes is

inhibited by Cs at a concentration of 1 ng/ml and T cell activation is inhibited at levels of 100 – 1000 ng/ml.[8] The results of this study revealed that Cs concentrations below 100 ng/ml in the aqueous humor cannot prevent corneal allograft rejection in a high risk rat model and showed that a Cs drug delivery system consisting of drug in a PLGA polymer is an effective means of achieving early high drug concentrations in the aqueous humor and is nontoxic in the anterior chamber of rats.

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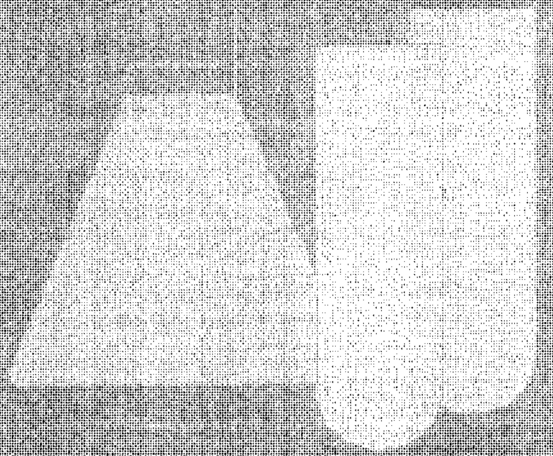
Chemical composition and functions of saliva



Dennis E. Lopatin, Ph.D.

PTO-000692

Chronology of defining salivary components and functions

- Beginning in 1950's whole saliva evaluated (antimicrobial properties, role in microbial attachment, mineralization, taste, lubrication)
- Secretions of major glands (parotid and submandibular/sublingual)
- In 1970's individual components isolated and biochemically characterized
- In mid-1980's beginning to map functional domains (peptide synthesis and recombinant approaches)

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Major salivary components

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Current concepts regarding the functional features of salivary macromolecules

- Recent structure/function studies have identified general principles regarding function
- Based on *in vitro* studies of purified molecules
- Additional studies required to evaluate concepts *in situ*

Dennis E. Lopatin, Ph.D.

Conformational requirements

- Conformation or shape of a molecule is critical for its biological function
- Examples
  - Proline-rich proteins interact with *A. viscosus* and *St. gordonii* only when adsorbed onto mineralized surface
  - Statherins and histatins require α-helical conformation
  - Human salivary amylase require 5 inter-chain disulfide bonds

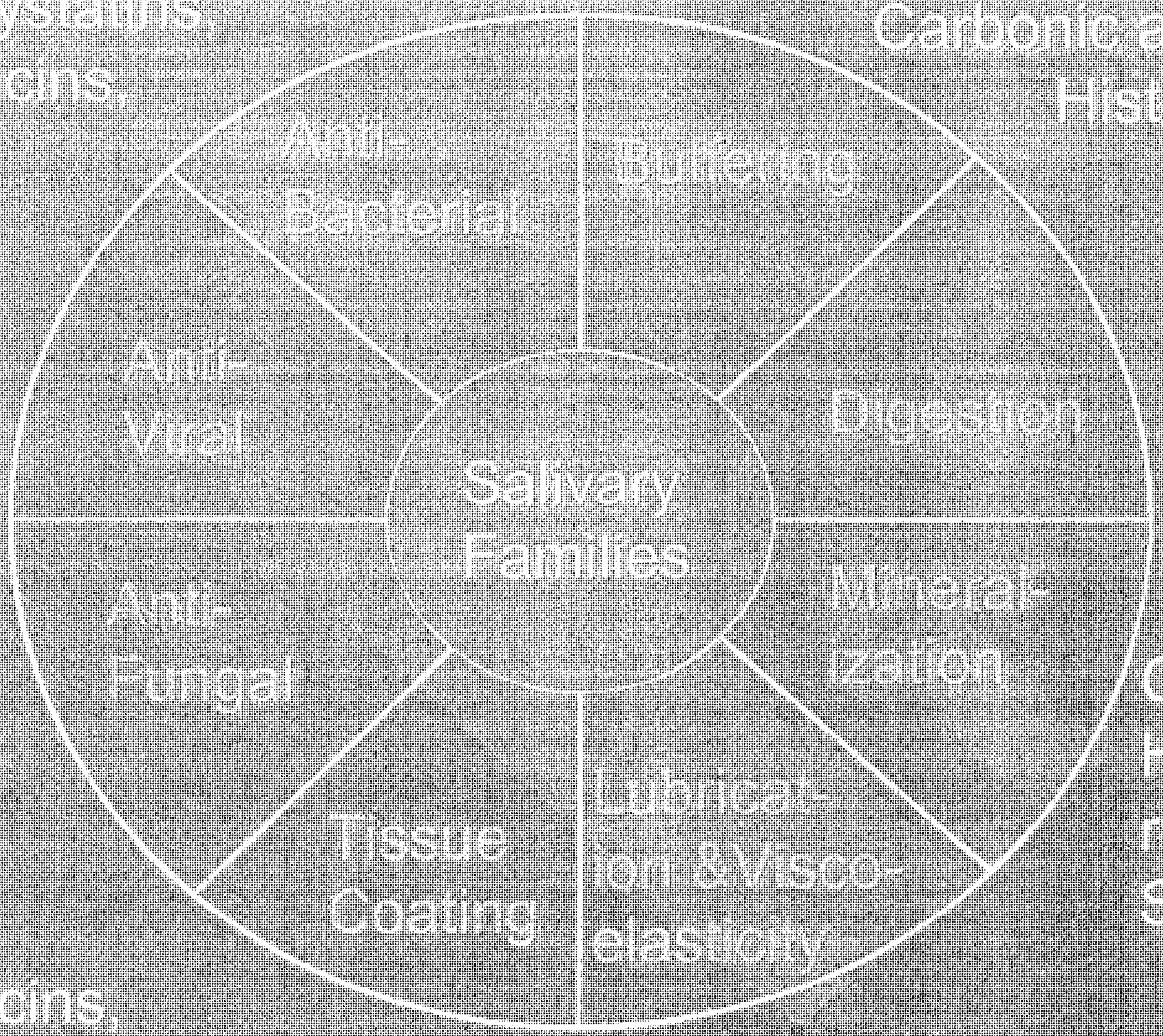
Multifunctionality

Amylases, Cystatins, Histatins, Mucins, Peroxidases

Carbonic anhydrases, Histatins

Cystatins, Mucins

Amylases, Mucins, Lipase



Histatins

Cystatins, Histatins, Proline rich proteins, Statherins

Amylases, Cystatins, Mucins, Proline-rich proteins, Statherins

Mucins, Statherins

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Page no. 6

adapted from M.J. Levine, 1993

Dennis E. Lopatin, Ph

Redundancy

- Saliva has built-in redundancy in regard to its protective functions.
- Example - Many salivary molecules can inhibit the precipitation of calcium phosphate salts.
  - strong inhibitors such as statherin and acidic proline-rich proteins
  - moderate inhibitors such as histatins and cystatins
  - weak inhibitors such as mucins and amylase

Amphifunctionality

- A molecule may have both protective and detrimental properties - "double-edged sword".
- May depend on molecule's location or site of action
  - Amylases
    - » In solution, they facilitate clearance of viridans streptococci
    - » Adsorbed to tooth surface, they can promote adherence of these bacteria and digest starch to dietary maltose and production of acid
  - Statherin and acidic proline-rich proteins
    - » At enamel surface, they play an important role in mineralization by inhibiting the formation of primary and secondary calcium phosphate salts. When adsorbed to the enamel surface, they promote attachment of cariogenic microorganisms.

Dennis E. Lopatin, Ph.D.

Complexing

- Functional relationships exist between different molecules in saliva
- Two types of complexing (covalent and non-covalent)
  - homotypic (between similar molecules)
  - heterotypic (between different molecules)
- Example: Mucins
  - homotypic complexes necessary for lubrication and viscoelastic properties
  - heterotypic complexes with sIgA, lysozyme and cystatins concentrate these anti-microbials at tissue interfaces

Dennis E. Lopatin, Ph.D.

Salivary Protein Functions

Oral function	Problem	Protein function
• Acts as an airway	• Air-born organisms • Dehydration	• Anti-bacterial systems • Water-retaining glycoproteins
• Speech	• Need for lubrication	• Lubrication system
• Taste	--	• Gustin
• Entry-point for food mastication, swallowing	• Food-born organisms • Soft and hard tissue abrasion • Food toxins	• Anti-bacterial systems • Lubrication; mucins, statherin • Toxin-neutralization

Salivary Protein Functions (cont'd)

Oral function	Problem	Protein function
• Control of in- digenous & in- vading bacteria, fungi and viruses	• Colonization & infection • Controlling pathogens and commensals • Adhesion of bac- teria versus their detection	• Anti-bacterial systems • Immunoglobu- lins, histatins, glycoproteins, lysozyme, sia- loperoxidase, lactoferrin • Adhesion- modulating pro- teins

Salivary Protein Functions (cont'd)

Oral function	Protein function
• Digestion	• Starch & fat hydrolysis: amylase and lingual lipase
• Protection & repair of oral tissues	• Mucin-rich protective barrier film • Protease inhibitors, cystatins: tissue growth factors
• Texture: carbohydrate dependent proteases	

Salivary Protein Functions (cont'd)

Oral function	Problem	Protein function
• Protection & repair of hard tissues	• Enamel mineral is potentially soluble: acid-damaged enamel requires remineralization	• Biologically controlled protective & repairative inorganic environment, stabilized by statherin, acidic proline-rich and pellicle proteins
• Pellicle formation	—	—
• Plaque acid formation	• Plaque pH control	• Basic amino acids & peptides

Dennis E. Lopatin, Ph.D

Mucins

- Lack precise folded structure of globular proteins
- Asymmetrical molecules with open, randomly organized structure
- Polypeptide backbone (apomucin) with CHO side-chains
- Side-chains may end in negatively charged groups, such as sialic acid and bound sulfate
- Hydrophillic, entraining water (resists dehydration)
- Unique rheological properties (e.g., high elasticity, adhesiveness, and low solubility)
- Two major mucins (MG1 and MG2)

Mucin Functions

- Tissue Coating
  - Protective coating about hard and soft tissues
  - Primary role in formation of acquired pellicle
  - Concentrates anti-microbial molecules at mucosal interface
- Lubrication
  - Align themselves with direction of flow (characteristic of asymmetric molecules)
  - Increases lubricating qualities (film strength)
  - Film strength determines how effectively opposed moving surfaces are kept apart

Mucin Functions (cont'd)

- Aggregation of bacterial cells
  - Bacterial adhere to mucins may result in surface attachment, or
  - Mucin-coated bacteria may be unable to attach to surface
- Bacterial adhesion
  - Mucin oligosaccharides mimic those on mucosal cell surface
  - React with bacterial adhesins, thereby blocking them

Amylases

- Calcium metalloenzyme
- Hydrolyzes α(1-4) bonds of starches such as amylose and amylopectin
- Several salivary isoenzymes
- Maltose is the major end-product (20% is glucose)
- "Appears" to have digestive function
- Why is it also present in tears, serum, bronchial, and male and female urogenital secretions?
- A role in modulating bacterial adherence?

Lingual Lipase

- Secreted by von Ebner's glands of tongue
- Involved in first phase of fat digestion
- Hydrolyzes medium- to long-chain triglycerides
- Important in digestion of milk fat in new-born
- Unlike other mammalian lipases, it is highly hydrophobic and readily enters fat globules

Scientific terms

- Calcium phosphate salts of dental enamel are soluble under typical conditions of pH and ionic strength
- Supersaturation of calcium phosphates maintain enamel integrity
- Stabilization or crystallization of supersaturated calcium phosphate in dental saliva and oral fluid
- Produced by acinar cells in salivary glands
- Also an effective lubricant

Dennis E. Liberatore, Ph.D.

Proline-rich Proteins (PRPs)

- Like statherin, PRPs are also highly asymmetrical
- Inhibitors of calcium phosphate crystal growth
- Inhibition due to first 30 residues of negatively-charged amino-terminal end
- Present in the initially formed enamel pellicle and in "mature" pellicles

Role of PRPs in enamel pellicle formation

- Acquired enamel pellicle is 0.1-1.0 μm thick layer of macromolecular material on the dental mineral surface
- Pellicle is formed by selective adsorption of hydroxyapatite-reactive salivary proteins, serum proteins and microbial products such as glucans and glucosyl-transferase
- Pellicle acts as a diffusion barrier, slowing both attacks by bacterial acids and loss of dissolved calcium and phosphate ions

Remineralization of enamel and calcium phosphate inhibitors

- Early caries are repaired despite presence of mineralization inhibitors in saliva
- Sound surface layer of early carious lesion forms impermeable barrier to diffusion of high mol.wt. inhibitors.
- Still permeable to calcium and phosphate ions
- Inhibitors may encourage mineralization by preventing crystal growth on the surface of lesion by keeping pores open

Calculus formation and calcium phosphate inhibitors

- Calculus forms in plaque despite inhibitory action of statherin and PRPs in saliva
- May be due to failure to diffuse into calcifying plaque
- Proteolytic enzymes of oral bacteria or lysed leukocytes may destroy inhibitory proteins
- Plaque bacteria may produce their own inhibitors

Calcium phosphate precipitation inhibitors and plaque

- Statherin and PRPs might be expected to occur in plaque, have not been detected
- Plaque bacteria produce calcium phosphate inhibitors
- Might be necessary to prevent calcification of bacteria - happens with dead cells
- Immobilized crystal growth inhibitors can function as nucleators of crystal growth
- Immobilization may occur in plaque, encouraging calculus formation

Interaction of oral bacteria with PRPs and other pellicle proteins

- Several salivary proteins appear to be involved in preventing or promoting bacterial adhesion to oral soft and hard tissues
- PRPs are strong promoters of bacterial adhesion
  - Amino terminal: control calcium phosphate chemistry
  - Carboxy terminal: interaction with oral bacteria
- Interactions are highly specific
  - Depends on proline-glutamine carboxy-terminal dipeptide
  - PRPs in solution do not inhibit adhesion of bacteria

These anti-microbial proteins will be discussed in a later lecture

- Secretory Immunoglobulins
- Lactoferrin
- Lysozyme
- Sialoperoxidase
- Cystatins
- Histatins

Summary - Clinical Highlights

- Understanding of salivary mechanisms at fundamental level a prerequisite for
  - effective treatment of salivary gland dysfunctions
  - modulation of bacterial colonization
  - development of artificial saliva other "cutting edge" approaches to salivary dysfunctions and diseases

Department of Biologic & Materials Sciences

BMS 513- Biology of the Salivary Glands 2001

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Lecture No.	Date	Instructor	Title of Lecture	PP
1	April 30, 2001 (Monday)	Lopatin	*Introduction to the Salivary Gland*	Do (6.
2	May 2, 2001 (Wednesday)	Lopatin	*Chemical Composition and Functions of Saliva*	Do (14
3	May 7, 2001 (Monday)	Nosrat	*Salivary Ion and Fluid Secretion (revised 5/7/01)*	Do (11
4	May 9, 2001 (Wednesday)	Nosrat	*Excitation-Secretion Coupling*	Do (4.
5	May 14, 2001 (Monday)		*No Class*	
6	May 16, 2001 (Wednesday)	D'Silva	*Cell Biology of Salivary Protein Secretion*	Do (5M
	May 21, 2001 (Monday)	Lopatin	*Non-Immune Anti-Microbial Activities in Saliva*	Do (ml
7	May 23, 2001 (Wednesday)		*Mid-Term Examination*	
	May 28, 2001 (Monday)		Memorial Day	
8	May 30, 2001 (Wednesday)	Lopatin	*Mucosal Immune System*	Do (1.

9	June 4, 2001 (Monday)	Sweier	*Aging and Diseases of the Salivary Glands*	Do (1.
10	June 6, 2001 (Wednesday)	Sweier	*Pharmacologically-Mediated Salivary Dysfunction and the Pharmacologic Management of Salivary Diseases*	Do (56
11	June 11, 2001 (Monday)	Lopatin	*Saliva as a Diagnostic Fluid*	Do (1.
12	June 13, 2001 (Wednesday)	Lopatin	*Future Directions in Salivary Gland Research*	Do (2.

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1997	1998	1999	2000	2001
Midterm	Midterm	Midterm	Midterm	Midterm
Final	Final	Final	Final	Final

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MidTerm
Combined Midterm/Final and Course Grade

Required Text

W.M. Edgar and D.M. O'Mullane (eds.). Saliva and Oral Health;London; British Dental Association; 1996.

Recommended texts

Bradley, R.M. Essentials of oral physiology. St. Louis; Mosby-Y Book; 1995. (You will need to purchase this book for Dr. Bradley's course in the You might want to buy it now.)

Dobrosielski-Vergona, K. (ed.) Biology of the salivary glands. E Raton; CRC Press; 1993.

Malamud, D. and Tabak, L. (eds.) Saliva as a diagnostic fluid. I York; Annals of the New York Academy of Sciences; Vol. 694, :

Additional Readings have been placed on reserve in the Dentistry Library.

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2000 AAPS ANNUAL MEETING

Influence of Three Emulsion Formulation Parameters on the Ocular Bioavailability of Cyclosporine A in Albino Rabbits

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Purpose. Assess the influence of 3 emulsion formulation parameters on concentrations of cyclosporine A (CsA) in rabbit cornea and conjunctiva, target tissues for the topical treatment of dry eye. **Methods**. Seven castor oil-in-water emulsions were made to contain (% w/w) 0.125 to 5 castor oil (CO), 0.1 to 3 polysorbate 80 (PS80), 0.01 to 0.4 CsA, 0.05 Carbomer 1342 (as secondary emulsifier), glycerin (for tonicity), and NaOH (for pH adjustment). Formulations were designed to evaluate: 1) Proportionality between tissue concentration and instilled dose at constant CsA/CO ratio and PS80 concentration, 2) Effect of CsA/CO ratio at constant CsA dose, and 3) Effect of globule size. Female albino rabbits received one 28.5 µL dose of CsA emulsion to each eye. Animals were euthanized at 0.5, 1.5, 3, 6, and 12 hr postdose, immediately after which cornea and conjunctiva were collected from each eye. CsA was extracted with methanol and then quantified by LC-MS/MS. **Results**. Corneal and conjunctival concentration-time profiles were steady between 0.5 and 12 hr. At a constant CsA/CO ratio of 7.4%, tissue concentrations increased nonlinearly with CsA dose to an asymptotic concentration of approximately 11,000 ng/g in cornea and 5,000 ng/g in conjunctiva. Decreasing the CsA/CO ratio to 1%, but keeping total CsA dose unchanged, decreased corneal and conjunctival concentrations by 65-75%. Reducing PS80 concentration from 3% to 1% increased mean globule diameter from 0.5 µm to 1.8 µm, but had no effect on ocular tissue concentrations. **Conclusions**. Increasing the CsA/CO ratio in CsA emulsions increases corneal and conjunctival bioavailability. Ocular tissue concentrations increase nonlinearly and are independent of globule size.

Expert Opinion

Monthly Focus: Endocrine & Metabolic

Primary Sjögren's syndrome: oral aspects on pathogenesis, diagnostic criteria, clinical features and approaches for therapy

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Primary Sjögren's syndrome (pSS) is a chronic inflammatory systemic autoimmune disease affecting the exocrine glands and predominantly the salivary and lacrimal glands. The impaired gland function is assumed to be a result of progressive lymphocyte-mediated destruction of the exocrine gland tissue leading to the cardinal manifestations, hyposalivation and keratoconjunctivitis sicca (KCS), as well as devastating symptoms of oral and ocular dryness. Although primarily characterised as an exocrine dysfunction, non-exocrine organs may also be affected. The onset and course of pSS is usually insidious but may develop into a disabling disease, which profoundly affects the patient's general well being and quality of life. Moreover, pSS may even evolve into a lymphoid malignancy. The aetiology of pSS remains unknown but the pathogenesis of exocrine cell damage is apparently multi-factorial, including immunological, genetic, hormonal and viral components. Recent research also includes neurogenic aspects of exocrine gland dysfunction, including the interference of immune mediators with glandular response to neurotransmitters released from nerve fibres. pSS usually affects middle-aged women and the female:male ratio is 9:1. The prevalence varies from 0.29 - 4.8%, depending on the population sampled and the diagnostic criteria used. At present, there are no specific diagnostic tests for pSS and no universally accepted diagnostic criteria. The current therapy is primarily symptomatic. This review focuses on the current oral clinical, diagnostic, pathogenic and therapeutic aspects of pSS.

Keywords: diagnostic criteria, labial salivary gland histopathology, primary Sjögren's syndrome, salivary gland function, therapy, xerostomia

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1. Introduction

Sjögren's syndrome (SS) is classified as a chronic inflammatory systemic autoimmune disease involving, in particular, the lacrimal and salivary glands [1]. Accordingly, the term 'autoimmune exocrinopathy' has been proposed [1]. The impaired gland function is suggested to be a consequence of lymphocyte-mediated destruction of the exocrine glands leading to KCS and hyposalivation and accordingly debilitating symptoms of ocular and oral dryness [2]. SS is classified into primary and secondary forms. pSS is characterised by the simultaneous presence of KCS and hyposalivation in patients not fulfilling internationally accepted criteria for another

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chronic inflammatory connective tissue disease, whereas the secondary form defines the disease entity in the presence of another chronic inflammatory connective tissue disease such as rheumatoid arthritis (RA) or systemic lupus erythematous [3]. The distinction reflects the fact that there are fundamental clinical, immunological and genetic differences between the two disease entities [4]. As in other chronic inflammatory connective tissue diseases, the exact aetiology and pathogenesis of SS remains obscure but appears to involve interactions between genetic, immunological, hormonal and environmental factors [5]. Recent research has focused on the neurogenic aspects of exocrine gland hypofunction in pSS and also on a more general dysregulation of the autonomic nervous system [6-8]. Clinically, pSS may exhibit a wide variety of disease manifestations due to involvement of additional exocrine as well as non-exocrine organs [9-11]. Occurrence of serum autoantibodies is also a well-recognised feature in pSS [12,13]. Furthermore, there is an increased risk of developing malignant lymphoma [14,15]. Since the aetiology and pathogenic events of pSS remain unclear, the diagnosis relies upon characteristic symptoms and clinical manifestations. However, the extent of gland dysfunction and additional clinical manifestations varies greatly among patients complicating establishment of the diagnosis of pSS, especially in those with vague symptoms. At present, there is no curative treatment for pSS and current topical and systemic agents mainly aim at alleviating symptoms. Early diagnosis is, however, crucial in order to prevent complications of exocrine gland hypofunction and to recognise more serious complications such as the development of malignant lymphoma. This review has its main focus on pSS and primarily discusses the oral aspects of the disease as well as the pharmacotherapy of the oral disease manifestations.

2. Demographic aspects

pSS predominantly affects middle-aged women but may debut at any age from early childhood to old age [16]. In children, pSS is a rare but probably also a frequently undiagnosed disease [17,18]. Diagnosis is often delayed 5 - 10 years, reflecting the fact that the onset of disease is insidious, presenting various and often non-specific symptoms [9,19]. The female:male ratio of occurrence is 9:1 and estimates of prevalence of pSS vary worldwide from 0.29 - 4.8% [20]. The wide range probably reflects differences in the population sampled and the diagnostic criteria used in the studies. Thus, it is not clear whether ethnic or geographic factors influence the prevalence of pSS, since use of various diagnostic criteria make studies incomparable. In a Swedish epidemiological study, using the Copenhagen criteria, the prevalence of pSS was 2.7% in a population between the age of 52 and 72 years [21]. In a recent Danish study of individuals between the age of 30 and 60, the estimate of prevalence of pSS was 0.2 - 0.8% according to the Copenhagen criteria and 0.6 - 2.1% according to the preliminary European criteria [22]. Thus, in Scandinavia, pSS is the most common disorder among the group of chronic systemic rheumatic diseases [20].

3. Aspects on diagnostic criteria of pSS

A global consensus concerning uniform criteria for the diagnosis of pSS has not yet been reached. The lack of uniformity not only leads to confusion among clinicians and patients but also complicates the fundamental important comparison of scientific data from different parts of the world. Several sets of criteria have been applied for the diagnosis of SS [23,24] of which five are commonly used worldwide: the Copenhagen criteria [3], the Californian criteria [25], the Greek criteria [26], the Japanese criteria [27] and the recent preliminary European criteria [28] (Table 1). Since the underlying cause of pSS remains unclear, the diagnosis relies upon combinations of symptoms, clinical features and laboratory abnormalities. At present, there is no single test or specific marker for oral, ocular or systemic involvement that can form the basis for a diagnosis of pSS. Accordingly, the various existing sets of diagnostic criteria are based on different combinations of tests and clinical features [23,24]. Some include subjective symptoms of dryness [25-28] and some serological parameters [25,28], whereas others only include objective measure for oral and ocular involvement [3]. The preliminary set of criteria defined by the European Community Study group (Table 1) is based upon computerised clinical data from 26 European SS centres [28]. The preliminary European criteria from 1993 include six items and the diagnosis of pSS is based on the presence of at least four [28]. It has been criticised that these criteria could be fulfilled in the absence of antinuclear antibody or labial salivary gland focal lymphocytic infiltrates and thus erroneously could classify patients with drug-induced oral and ocular dryness and secretory hypofunction as pSS [23,24]. However, a thorough anamnesis will often reveal medications with potential hyposalivatory side effects. The preliminary European criteria are currently and widely being evaluated in order to validate each item [28,29]. In this process, is has been proposed that a revision of the European criteria should require inclusion of focus score ≥ 1 and/or presence of anti-Ro/SSA/anti-La/SSB serum autoantibodies among four of the six items which have to be met [30,31]. Since our current knowledge concerning pSS disease course and development is limited requiring presence of focal lymphocytic infiltrative changes and serum autoantibodies may prevent diagnosis and thus intervention of early stages of pSS. Although, of fundamental importance for proper diagnosis of SS, only the Californian and European criteria operate with pre-existing lymphoma, AIDS, graft *versus* host disease or sarcoidosis as exclusions criteria.

3.1 Diagnosis of the oral component of pSS

Salivary gland dysfunction is evaluated by means of various objective tests including parotid sialography, salivary gland scintigraphy, sialometry and labial salivary gland biopsy. The preliminary European criteria also include questionnaire concerning symptoms of oral dryness [28].

Table 1. Presentation of the five most commonly used criteria sets for diagnosing pSS.

	Copenhagen	Californian	Greek	Japan	European
Oral component					
Symptoms of oral dryness		+	+	+	+
Parotid enlargement			+	+	
Unstimulated whole sialometry	≤ 1.5 ml/15 min				≤ 1.5 ml/15 min
Parotid sialometry		Decreased[§]	≤ 1 ml/5 min/gland		
Sialography				+	+
Scintigraphy	+				+
Labial salivary gland biopsy	Focus score > 1	Focus score ≥ 2	Focus score ≥ 2+	Focus score > 1	Focus score ≥ 1
Ocular component					
Symptoms of ocular dryness			+	+	+
Schirmer-I test	≤ 10 mm/5 min	< 9 mm/5 min	≤ 5 mm/5 min	< 10 mm/5 min	≤ 5 mm/5 min
Break-up time	≤ 10 sec				
Rose Bengal staining	≥ 4 points	+	+	+	≥ 4 points
Fluorescein staining		+		+	
Lacrimal gland biopsy				+	
Serological component					
Anti-Ro/SSA or -La/SSB		Positive or			Positive
ANA		Titre ≥ 1:160 or			Positive
RF		Titre ≥ 1:160			Positive

Exclusion criteria for the Californian and European criteria: existing lymphoma, AIDS, sarcoidosis or 'graft *versus* host disease'.

[§]Value not specified.

+: Test or manifestation required but not further specified; ANA: Antinuclear antibodies; pSS: Primary Sjögren's syndrome; RF: Rheumatoid factor.

3.1.1 Parotid sialography

Sialography is a radiological method based on retrograde injection of contrast media into the parotid duct providing visualisation of anatomical changes of the salivary gland duct system [32]. Sialectasis caused by dilatations of the peripheral parts of the duct system is the most typical finding in SS [33-35]. The sialographic findings are not specific to SS and may be indistinguishable from those associated with chronic sialadenitis of other causes [36,37]. Moreover, sialectasis had been found in 15 - 20% of a normal population [36]. On the other hand, it has been shown that the pathological findings of parotid sialography are more sensitive than the histopathological changes of labial salivary gland biopsy in the diagnosis of SS [38]. Thus, sialography may identify some pSS patients with negative labial salivary gland biopsy [38,39] but may also fail to detect patients with known SS [33,34]. Parotid sialography is a less sensitive indicator of the secretory ability that parotid flow rate measurement [34] and correlates poorly with parotid gland histopathology [9]. Oil-based contrast media provide a more sensitive indication of salivary gland tissue changes [9,35,40] than the water-based media [34,35]. However, abnormal salivary glands may retain the oil-based contrast media causing infection or granulomatuos reactions [36].

3.1.2 Salivary gland scintigraphy

Salivary gland scintigraphy is a relatively non-invasive test. It provides a functional assessment of the major salivary glands by analysing the rate of glandular radioisotope uptake and the following excretion into the oral cavity after iv. injection of ^{99m}Technetium-sodium pertechnetate ($^{99m}TcO_4^-$) [41]. $^{99m}TcO_4^-$ is believed to substitute the chloride ion and to be transported transcellularly *via* chloride transport systems such as the sodium/potassium/chloride co-transport system, the chloride/bicarbonate exchange system and chloride channels in the plasma membranes of glandular cells [41]. The advantages of using scintigraphy are the simultaneous examination of the activity of the major salivary glands and the possibility to evaluate the functional capacity of each gland under unstimulated as well as stimulated conditions [39,42-44]. Glandular uptake of the radiolabelled isotope reflects the remaining functional parenchyma and loss of the isotope demonstrates the ability of the major salivary glands to secrete saliva [45]. The response of the salivary glands to gustatory stimulation with citric acid reflects the secretory capacity of the major salivary glands [45]. In pSS, salivary scintigraphy shows diminished $^{99m}TcO_4^-$ uptake to the gland or delayed/absent loss following citric acid stimulation [43,44]. Although not specific to pSS, the abnormal salivary scintigraphic changes correlate significantly with abnormal sialometric values of unstimulated and stimulated whole saliva as well as stimulated parotid saliva [43-46]. This indicates that measurements of salivary flow rates provide almost the same functional information of the major salivary glands, as does the scintigraphy. Furthermore, in contradiction to measurement of salivary flow rates (sialometry), salivary gland scintigraphy is rather expensive, requires isotope equipment and therefore can only be carried out in specialised departments with this special equipment. More recent non-invasive imaging tests for studying the major salivary glands include magnetic resonance imaging [47], ultrasound examination [48] and computerised tomography [49]. However, the clinical relevance of these tests for the diagnosis of pSS is still not clarified and awaits further investigation.

Table 2. Examples of causes of xerostomia and hyposalivation.

Medication	Antihypertensives, antidepressants, antipsychotics and antihistamines.
Systemic diseases	Conditions causing dehydration, Sjögren's syndrome, rheumatoid arthritis, sarcoidosis, dysregulated diabetes mellitus, HIV-infection and graft *versus* host-disease.
Irradiation	Radiation therapy of tumours in head and neck region.
Psychogenic disorders	Depression and anxiety
Factors affecting the autonomic outflow pathway	Autonomic dysfunction (e.g., Holme-Adies syndrome), brain tumours, cerebral-vascular diseases and neurosurgical traumas affecting the peripheral nerves and the central nervous system (e.g., the trigeminal, facial or glossopharyngeal nerves and nuclei salivatorii, respectively).
Local factors	Salivary gland infection and obstruction.

3.1.3 Sialometry

Salivary flow rates, unstimulated or stimulated, can be measured for whole saliva or for separate secretions from the parotid or submandibular/sublingual glands. However, salivary flow rates are subject to physiological variation and may exhibit a wide range of values between individuals [50,51]. Thus, it is of crucial importance to use a standardised, reproducible test technique.

Measurement of unstimulated whole saliva provides a general assessment of salivary gland capacities under basal conditions. Unstimulated whole salivary flow rates are more closely associated with symptoms of xerostomia than the stimulated whole salivary flow rates [52,53]. In the diagnosis of SS, an unstimulated whole salivary flow rate of ≤ 1.5 ml/15 min is considered pathological and designated hyposalivation [3]. This test has been found to be highly reproducible [19] as well as having a high diagnostic specificity (80.7%) for pSS [54]. Furthermore, whole unstimulated salivary flow rate of < 0.10 ml/min is highly correlated to symptoms of xerostomia in pSS [53,55]. Unstimulated whole salivary flow rates appear to be significantly lower (i.e., < 0.05 ml/min) in pSS patients with focal lymphocytic infiltrates and/or serum autoantibodies than those without these findings [56]. The salivary gland hypofunction has been considered more severe in pSS than that seen in secondary SS [57] but this is not supported by results of a recent study [58]. The diagnostic sensitivity and specificity of the measures of paraffin-chewing stimulated whole salivary flow rates, with a pathological cut-off value of ≤ 3.5 ml/5 min [3], are low and accordingly not included in the European criteria [28].

Separate parotid gland secretion can be measured with a Lashley cup that fits over the opening of the parotid duct using gustatory (citric acid) stimulation [25]. The maximal secretory rate is, however, directly proportional to the size of the gland and varies widely among individuals [59]. The diagnostic sensitivity and specificity of stimulated parotid salivary flow rate has been found to be rather low [28]. Furthermore, it has been shown that 45% of patients with pSS had normal parotid flow [60] and some studies reported reduced chewing stimulated whole saliva flow rates in spite of normal stimulated parotid flow rates [53,61]. Nevertheless, the test is included in the Californian criteria for pSS [25].

Measurement of whole salivary flow rate (sialometry) is a simple, reproducible and non-invasive test. It provides useful quantitative information concerning the salivary gland function in SS, although low salivary flow rates are not specific to pSS or any other disease (Table 2). Xerostomia is a cardinal symptom in pSS [9,61] but also a common complaint among medicated and diseased population groups and particularly among the elderly [62,63]. According to a Swedish population study, 35% in the age between 52 - 72 years complained of dry mouth [21]. In healthy individuals, however, there seem to be no overall diminution in salivary gland function with increased age [53,64,65]. Thus, although xerostomia is often related to salivary hypofunction [63], it may occur in the presence of normal salivary secretion [51,66] indicating that also the quality of saliva may be of importance to oral comfort.

3.1.4 Sialochemistry

The easy accessibility of salivary glands and their secretion have increased the interest in sialochemical analysis as a diagnostic tool. Since salivary glands are considered to be the target organs of inflammatory infiltration, sialochemical analysis appears to be useful in the evaluation of the influence of pSS on salivary gland function. Compositional changes in saliva are frequently found but until now none have been shown to be unique to pSS [67,68]. A major problem with most sialochemical studies is that almost all salivary constituents show great variations, even for healthy persons, and that concentrations of various constituents are not compared between individuals with the same flow rate [67,68]. Moreover, the use of saliva as a diagnostic aid for pSS is complicated by the fact that salivary glands may be affected differently in each individual. Ideally, in patients with pSS, histopathological and sialochemical analysis should be performed on the same glands and compared to age- and gender-matched healthy controls or other diseased controls with the same secretory rates. In this regard, measurement of labial salivary flow rates may be a future valuable diagnostic tool as it was recently demonstrated that SS patients have reduced labial salivary gland flow rates [69]. In pSS, salivary hypofunction is considered to be a result of loss of acinar cell function due to a

chronic and progressive infiltration of the gland tissue by lymphocytes. However, in histological specimens the duct epithelium appears unaffected. Compositional changes of saliva may be caused by changes in the acinar cells' secretion of electrolytes and fluid, functional alterations of the salivary ducts and disturbance of exocrine protein synthesis and release processes. Thus, saliva Na^+ and K^+ concentrations may be reflective of both acinar and ductal function. In pSS, increased concentrations of Na^+ and Cl^- have been reported, which is suggestive of impaired Na^+ and Cl^- reabsorptive capability of the ductal cells [53,70,71].

The chronic and progressive inflammatory infiltration of the salivary glands in pSS may alter the protein composition of their secretions. However, sialochemical studies have shown varying results [67,68]. Mucins in saliva are responsible for the salivary viscosity and function as lubricants of the oral mucosa. In this context, it is of particular interest that the inflammatory process of the salivary glands alters the quality of mucins produced since sialyl, fucosyl, galactosyl and galactosaminosyl residues and N-linked glycoconjungates are abnormal in the saliva of patients with SS [72]. Statherin and acidic proline-rich proteins (PRPs) have been used as markers of salivary protein secretion reflecting the secretion of selected parotid proteins as opposed to the total protein concentration [53]. Statherin has been found in the secretions from the human parotid, submandibular and von Ebner's glands but not in labial gland saliva [73]. PRPs are also secreted by serous acinar cells [74], although the concentrations of statherin and PRPs in stimulated parotid saliva have been found to be low with no significant differences between age- and gender-matched healthy controls and patients with pSS [53]. In spite of the fact that it has not yet been possible to show a direct relationship between separate constituents of saliva and risk of developing oral diseases, maintenance of oral health may be related to the output of saliva and its composition.

3.1.5 Labial salivary gland biopsy

Most criteria for the diagnosis of pSS advocate the use of lower labial salivary gland biopsy as an important diagnostic tool and it is considered the most reliable objective criterion for defining salivary gland involvement [52]. The histological findings of the labial salivary glands that have been given diagnostic significance are characterised by focal, periductal infiltration of lymphocytes and few plasma cells, adjacent to normal appearing parenchymal structures [52] (Figure 1). The histopathological lesion is denoted focal sialadenitis. More non-specific histological changes such as diffuse inflammation, acinar atrophy, fibrosis and ductal dilatation and hyperplasia may also appear. In more extensive lesions, the gland tissue may exhibit pronounced degenerative changes in which the lymphocytic infiltrates have replaced the acinar tissue [52,75]. The degree of lymphocytic infiltration is evaluated semi-quantitatively by means of a focus scoring system in which a focus is defined as an aggregate of more than 50 lymphocytes per 4 mm^2 of glandular tissue [75]. A focus score > 1 is considered strongly indicative of SS [52,75]. However, according to the European criteria the presence of a focus score of 1 had the best balance between sensitivity (86.2%) and specificity (82.4%) [54]. There is still a considerable controversy about the value and necessity of labial salivary gland biopsy and its accuracy in the diagnosis of pSS. Thus, some SS patients do not exhibit significant focal lymphocytic infiltration of their labial salivary glands [54,56,76,77]. However, the histological evaluation of labial salivary gland biopsy only represenst a static picture and does not reflect possible temporal fluctuation of disease activity. Failure to detect lymphocytic infiltrates may not only reflect the fact that the disease affects different exocrine glands in different patients but also that smoking patients have a lower frequency of focal lymphocytic infiltrates [78]. Studies have clearly shown that focal lymphocytic infiltrates of the labial salivary glands are not specific for pSS but can also be observed in healthy individuals [56,79-81]. Hence, the presence of focal lymphocytic infiltrates in labial salivary glands of healthy individuals of all ages has been demonstrated in a range of 7 – 32% [56,79,80,82,83]. More recently, it has been shown that the prevalence of focal lymphocytic infiltrates increased significantly with age in the palatinal but not in the labial salivary glands [81]. Also, in human submandibular glands the prevalence of focal lymphocytic infiltrates increased in healthy individuals with ageing [84,85]. It is well-known that histological changes of the salivary glands including acinar atrophy, fibrosis, fatty degeneration, duct dilatation, as well as various degrees of diffuse and focal inflammation occur with increasing age [82,83,86], whereas the salivary gland function appears to be unaffected by age [56,64,87]. On the other hand, these histological changes may also be indicative of previous transient inflammation of which the degenerative changes are a permanent result [88]. It is not fully demonstrated whether atrophy, fibrosis and degeneration are consequences of long-standing pSS. However, focal lymphocytic infiltrates have been shown to increase over time, although no simultaneous decrease in stimulated whole saliva flow was found [89]. Moreover, the presence of focal lymphocytic infiltrates in labial salivary glands have been demonstrated among patients with myasthenia gravis [90], primary biliary cirrhosis [91], diabetes mellitus [92] and SOX syndrome [93]. Symptoms, signs and salivary gland histopathology resembling pSS have also been described in patients with acute and chronic graft *versus* host disease [94], AIDS [95], leukaemia or lymphoid malignancies [9,10] and sarcoidosis [96], disorders which are among exclusions criteria in both the Californian and European criteria for the diagnosis of SS [25,28]. However, other diseases like hepatitis C, fibromyalgia and chronic fatigue syndrome may clinically be difficult to distinguish from pSS [97,98]. Generally, traditional parotid gland biopsy is only performed in case of persistent, firm enlargement due to the risk of damaging the facial nerve and scarring of the skin. Parotid fine needle biopsy is routinely used in some clinics but there are, however, limitations on what can be deduced from smears prepared from an aspirate.